PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/47, C12N 15/85, C07K 16/18, 16/32, C12N 5/10, A61K 38/17, G01N 33/577, C12Q 1/68, A61K 39/395

A1

(11) International Publication Number: WO 98/13489

(43) International Publication Date: 2 April 1998 (02.04.98)

(21) International Application Number: PCT/EP97/05198

(22) International Filing Date: 22 September 1997 (22.09.97)

(30) Priority Data:
08/718,661 23 September 1996 (23.09.96) US

(71) Applicants (*for all designated States except US*): MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Berlin (DE). CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE [FR/FR]; Rue de la Cardonille, F-34094 Montpellier Cedex 05 (FR).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): SPENGLER, Dietmar [DE/DE]; Ungererstrasse 65, D-80805 München (DE). JOURNOT, Laurent [FR/FR]; 15, avenue de la Gare, F-34570 Pignan (FR).

(74) Agent: VOSSIUS & PARTNER; Siebertstrasse 4, D-81675 München (DE).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NUCLEIC ACID MOLECULES CODING FOR TUMOR SUPPRESSOR PROTEINS AND METHODS FOR THEIR ISOLATION

(57) Abstract

Described are novel proteins having the biological activity of a tumor suppressor protein and nucleic molecules coding for such proteins. Methods for the isolation of nucleic acid molecules encoding tumor suppressor proteins as well as nucleic acid molecules obtainable by said method are also provided. Further, vectors comprising said nucleic molecules wherein the nucleic acid molecules are operatively linked to regulatory elements allowing expression in prokaryotic or eukaryotic host cells can be used for the production of polypeptides encoded by said nucleic acid molecules which have tumor suppressor activity. Pharmaceutical and diagnostic compositions are provided comprising the nucleic acid molecules of the invention and/or comprising a nucleic acid molecule which is complementary to such a nucleic acid molecule. Described are also compositions which comprise polypeptides encoded by the described nucleic acid molecules which have tumor suppressor activity and/or an antibody specifically recognizing such polypeptides.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

NUCLEIC ACID MOLECULES CODING FOR TUMOR SUPPRESSOR PROTEINS AND METHODS FOR THEIR ISOLATION

The present invention relates to novel nucleic acid molecules coding for a protein having the biological activity of a tumor suppressor protein. The present invention also provides methods for the isolation of nucleic acid molecules encoding tumor suppressor proteins as well as nucleic acid molecules obtainable by said method. Further, the invention provides vectors comprising said nucleic acid molecules wherein the nucleic acid molecules are operatively linked to regulatory elements allowing expression in prokaryotic or eukaryotic host cells as well as polypeptides encoded by said nucleic acid molecules which have tumor suppressor activity and methods for their production. The present invention further relates to pharmaceutical and diagnostic compositions comprising the aforementioned nucleic acid molecules and/or comprising a nucleic acid molecule which is complementary to such a nucleic acid molecule. Described are also compositions which comprise polypeptides encoded by the described nucleic acid molecules which have tumor suppressor activity and/or an antibody specifically recognizing such polypeptides.

A network of genes, including cell cycle regulatory genes, proto-oncogenes, and tumor suppressor genes have emerged, which play major roles in normal physiological processes as well as in tumor progression (Grana and Reddy, Oncogene 11 (1995), 221-219; Hartwell and Kastan, Science 266 (1994), 1821-1828; Hoffman and Liebermann, Oncogene 9 (1995), 1807-1812; Sherr, Cell 79 (1994), 551-555). Oncogenes have first captured the lion's share of attention in

the molecular and genetic studies on cell transformation. But it has now been realized that there is an equally important second side of the coin, presented by a distinct class of genes known variously as tumor suppressor genes (TSGs) or anti-oncogenes. Logic dictates that there must exist an equally elaborate array of growth-constraining elements in the cell's signaling circuitry that serve to counteract the growth-promoting proto-oncogenes (Fisher, Cell 78 (1994), 539-542; Karp and Broder, Nature Med. 1 (1995), 309-320; Liebermann et al., Oncogene 11 (1995), 119-210; Thompson, Science 267 (1995), 1456-1462). These tumor suppressor genes are of special interest since they may open up new possibilities for the treatment of cancers of various kinds and may help to better understand the molecular mechanisms responsible for the development of cancer.

The isolation of such suppressor genes has become feasible by progress in various fields with major contributions of molecular genetics and cell cycle analysis. Molecular genetics applied linkage studies to the isolation of TSGs, but the most fruitful strategies have evolved from the study of the genetic mechanisms employed by nascent tumor cells to discard their second, surviving copy of a tumor suppressor gene which results in homozygosity at the tumor suppressor locus. This event can often be traced by following the fate of anonymous DNA markers whose polymorphism allows detection of hetero- and homozygous states in these chromosomal regions. By this strategy the identification of the retinoblastoma gene product (Rb), the Wilms tumor suppressor gene (WT) and the von Hippel-Lindau tumor-suppressor gene has been possible. Most recently the cloning of the breast cancer susceptibility genes, BRCA1 and BRCA2 (Miki et al., Science 266 (1995), 66-71; Wooster et al., Nature 378 (1995), 789-792) has been accomplished by this approach.

Yet, the vast majority of human cancers, including breast cancer, develop spontaneously or under poorly defined criteria of genetic susceptibility preventing linkage studies to perform and indicating that epigenetic mechanisms appear to play the major role in the initiation and formation of tumors, which seem to develop in a multi-step process.

Further support for the concept of TSGs came up with the characterization and isolation of the regulatory components of the mammalian cell cycle. This progress has led to the identification of a new class of candidate tumor suppressor genes, the ubiquitously expressed cyclin-dependent kinase inhibitors (cdk), which negatively regulate cell cycle progression. Among the various forms described so far (p15, p16, p18, p21 and p27) the cdk p16 has been demonstrated to be mutated in-vivo in a spectrum of tumors examined (Marx, Science 264 (1994), 344-345; Kamb et al., Science 264 (1994), 436-440; Nobori et al., Nature 368 (1994), 753-756).

Another important example of a tumor suppressor gene is the p53 TSG, whose biological activity has been elucidated in-vitro through molecular and biochemical studies before it became identified as the genetic cause of the Li-Fraumeni syndrome. It is one of the most frequently mutated tumor suppressor genes in human tumors from various origins (Hollstein et al., Science 253 (1991), 49-53). This TSG encodes a transcription factor with two important functional properties contributing to its growth-suppression function: induction of apoptosis and cell cycle arrest (Vogelstein and Kinzler, Cell 70 (1992), 523-526; Oren, FASEB J. 6 (1992), 3169-3176; Perry, Curr. Opin. Genet. Dev. 3 (1993), 50-54; Bates and Vousden, Curr. Opin. Genet. Dev. 6 (1996), 12-19).

Although tumor suppressor genes have recently attracted a lot of attention due to the possibility that they may provide important targets in the treatment of cancer, only a limited number of TSGs could be identified and cloned. Thus, there still exists a need for the identification of further tumor suppressor genes in order to better understand the mechanisms of the development of diseases such as cancer and to be able to provide means for the treatment of further forms of tumorous diseases or for the improved treatment of tumorous diseases. One reason for the slow progress in cloning TSGs may be seen in the fact that there exists no method for the identification and isolation which can be easily carried out in-vitro and allows the rapid screening of a plurality of potential sequences for tumor suppressor activity.

Thus, the technical problem underlying the present invention is to provide further nucleic acid molecules coding for proteins displaying tumor suppressor activity as well as methods for their identification and isolation.

The solution to the above technical problem is achieved by providing the embodiments characterized in the claims. Namely, nucleic acid molecules coding for a novel class of tumor suppressor proteins have been identified. This has been achieved by using an in-vitro functional expression transductory cloning technique. The described novel class of tumor suppressor proteins shares the ability of p53 to inhibit growth of tumor cells by controlling apoptotic cell death and cell cycle progression and appears to play a critical role in apoptosis and cell cycle regulation. However, the newly identified tumor suppressors display a more restricted pattern of tissue expression and distinct activities compared to known TSGs such as p53.

Thus, in one aspect, the present invention relates to a nucleic acid molecule encoding a protein having the biological activity of a tumor suppressor selected from the group consisting of:

(a) nucleic acid molecules coding for a polypeptide comprising the amino acid sequence given in SEQ ID NO.2;

(b) nucleic acid molecules comprising the nucleotide sequence given in SEQ ID NO.1;

(c) nucleic acid molecules hybridizing to a nucleic acid molecule as defined in (a) or (b); and

(d) nucleic acid molecules, the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of a nucleic acid molecule as defined in (a), (b) or (c).

The nucleic acid molecule with the nucleotide sequence of the coding region as depicted in SEQ ID NO. 1 codes for a protein of 667 amino acids with a predicted molecular weight of 75 kDa (Figure 1A). The ATG of AGGCC<u>ATG</u>G (SEQ ID NO.

4) was assigned as initiation codon on the basis of its close match to the CC(A/G)CCATGG (SEQ ID NO. 5) Kozak consensus sequence for favored initiation of translation and the presence of an in-frame TGA stop codon 12 nucleotides upstream. Data base searches revealed the presence of seven zinc fingers (Klug and Schwabe, FASEB J. (1995), 597-604) in the N-terminal region. However, homologies to other members of the zinc finger protein family were low (30% for the best), with the closest group being the GLI-Krüppel family of zinc finger proteins which have been implicated in normal development and tumor formation (Ruppert et al., Mol. Cell. Biol. 8 (1988), 3104-3113). In particular, the first H/C link (HSRERPFKC (SEQ ID NO. 6)) is in good agreement with the consensus motif for the GLI-Krüppel family (H(S/T)GEKP(F/Y)XC (SEQ ID NO. 7)) (Schuh et al., Cell 47 (1986), 1025-1032). On the other hand, the remaining 459 C-terminal amino acids displayed no significant homologies to sequences in the Swissprot and NBRF-PIR data bases. The central region of the protein (275-383) is characterized by 34 PLE, PMQ or PML repeats, suggestive of a structure known as poly proline type II helix which is considered to be critically involved in protein-protein interactions (Williamson, Biochem. J. 297 (1994), 249-260). The COOH-terminal region is particularly P-, Q- and E-rich, a feature often displayed by transactivation domains of transcription factors. In addition, the presence of a putative phosphorylation site (H<u>S</u>PQK (SEQ ID NO. 8)) for cyclin-dependent kinases (Cdks) located between the second and third zinc finger motif (residues 56-60) as well as a putative protein kinase A (PKA)-phosphorylation site (KKW<u>T</u> (SEQ ID NO. 9)) at the very C-terminus (residues 663-666) suggests possible regulation by protein kinases.

Studies which had been carried out in the scope of the present invention revealed that the protein encoded by the nucleic acid sequence of SEQ ID NO. 1 displays the biological activity of a tumor suppressor.

The term "tumor suppressor", as used herein, relates to any protein/polypeptide inhibiting growth of tumor cells in-vitro and/or in-vivo. Growth inhibition involves mechanisms such as control of apoptosis and/or of cell cycle progression as well

as mechanisms unidentified so far. "Tumor suppressors" are proteins displaying biological activities identical to or similar to those of p53, Rb (retinoblastoma gene product), WT (Wilms tumor suppressor gene), VHL (von Hippel-Lindau tumor suppressor gene), BRCA1 (breast cancer susceptibility gene) and p16 (cyclin-dependent kinase inhibitor).

Examples for important biological activities of a tumor suppressor are the capability to inhibit in-vitro proliferation of tumor cells as evidenced for instance by measuring colony formation, growth rate and cloning in soft agar as well as the capability to inhibit in-vivo tumor formation in nude mice. These biological activities can be determined, for example, according to Zhou et al., Proc. Natl. Acad. Sci. USA 91 (1994), 4165-4169; Chen et al., Science 250 (1990), 1576-1580; Baker et al., Science 249 (1990), 912-915; Diller et al., Mol. Cell. Biol. 10 (1990), 5772-5781; Casey et al., Oncogene 6 (1991), 1791-1797; Cheng et al., Cancer Research (1992), 222-226; Wang and Prives, Nature 376 (1995), 88-91; Mercer et al., Proc. Natl. Acad. Sci. USA 87 (1990), 6166-6170; Antelman et al., Oncogene 10 (1995), 697-704 or as described in the appended examples.

The protein encoded by the nucleic acid sequence of SEQ ID NO. 1 displays the ability to suppress tumor cell proliferation which could be demonstrated by the constitutive and induced expression of said protein in transfected tumor cells. Furthermore, said protein is capable of inhibiting anchorage-independent growth which is often correlated with tumorigenesis and is a strong criteria for cultured cell transformation. Furthermore, this novel protein is able to suppress tumor formation of transformed cells injected in nude mice. Thus, the protein of the invention displays all essential features of a tumor suppressor similar to those of, for example, p53. This new tumor suppressor is also able to induce apoptosis resulting in inhibition of tumor cell growth. However, this new tumor suppressor exhibits functional differences compared to p53, for instance the induction of apoptotic c ll death is more pronounced in Saos-2 cells for the protein of the invention than for p53. Furthermore, the tumor suppressor of the invention induces G1 arrest of the cell cycle, in contrast to p53, independently from the

transactivation of the gene encoding the cyclin-dependent kinase inhibitor p21. Finally, it had been shown that this protein acts as nuclear transcription factor.

From the above it is evident that the nucleotide sequence depicted in SEQ ID NO. 1 codes for a novel class of tumor suppressors. By the provision of this nucleotide sequence it is now possible to isolate identical or similar nucleic acid molecules which code for proteins with the biological activity of a tumor suppressor from other species or organisms. Well-established approaches for the identification and isolation of such related sequences are, for example, the isolation from genomic or cDNA libraries using the complete or part of the disclosed sequence as a probe or the amplification of corresponding nucleic acid molecules by polymerase chain reaction using specific primers.

Thus, the invention also relates to nucleic acid molecules which hybridize to the above described nucleic acid molecules and differ at one or more positions in comparison to these as long as they encode a protein having tumor suppressor activity. Such molecules comprise those which are changed, for example, by deletion(s), insertion(s), alteration(s) or any other modification known in the art in comparison to the above described nucleic acid molecules. Methods for introducing such modifications in the nucleic acid molecules according to the invention are well-known to the person skilled in the art; see, e.g., Sambrook et al. (Molecular cloning; A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989)). The invention also relates to nucleic acid molecules the sequence of which differs from the sequence of any of the above-described molecules due to the degeneracy of the genetic code.

With respect to the sequences characterized under (c) above, the term "hybridizing" in this context is understood as referring to conventional hybridization conditions, preferably such as hybridization in 50%formamide/6xSSC/0.1 %SDS/100µg/ml ssDNA, in which temperatures for hybridization are above 37°C and temperatures for washing in 0.1xSSC/0.1%SDS

are above 55°C. Most preferably, the term "hybridizing" refers to stringent hybridization conditions, for xample such as described in Sambrook, supra.
Nucleic acid molecules which hybridize to molecules according to the invention and encode a tumor suppressor may, for example, be those which code for proteins in which putative phosphorylation sites are altered. Biochemical analysis of the regulation of wild-type p53 sequence-specific DNA binding has, for instance, shown that the unphosphorylated tetramer has a cryptic sequence-specific DNA binding activity. This cryptic or latent state of p53 depends upon a C-terminal negative regulatory domain, which locks the unphosphorylated tetramer in an inactive state. Phosphorylation of the C-terminal negative regulatory domain of latent p53 by either protein kinase C or casein kinase II or deletion of the regulatory domain activates sequence-specific DNA binding. In addition, a monoclonal antibody can mimic the effects of protein kinases and activate latent p53. Thus, neutralization of this negative regulatory domain by covalent or non-covalent modification is an important stage in the activation of p53. As described above, the protein encoded by SEQ ID NO. 1 has two putative phosphorylation sites for protein kinases. A consensus motive for cyclin-dependent kinases is located in the DNA-binding domain raising the possibility that the DNA-binding affinity can be finetuned by the driving forces of the cell cycle, constituting a putative feedback loop. This regulatory site could offer the interesting possibility that molecular tools directed against cyclin/cyclin-dependent kinase can via this loop modify the activity of this protein and implement apoptosis proficiency to target tissues. Similarly the presence of a motif for protein kinase A at the very C-terminus of the protein transactivations domain could be a critical target to modulate transactivation potency.

The above-described nucleic acid molecules which encode a novel class of tumor suppressors had been identified by using an in-vitro functional transductory cloning technique. Thus, in another aspect, the present invention relates to a process for the identification and cloning of nucleic acid molecules encoding a

protein having the biological activity of a tumor suppressor comprising the steps of:

(i) transfecting mammalian cells with

(a) a first vector comprising a scorable reporter gene operatively linked to regulatory elements comprising at least one cAMP responsive element so located relative to said reporter gene to permit cAMP inducible expression thereof; and

(b) pools of expression vectors comprising nucleic acid molecules linked to regulatory elements allowing expression in the mammalian cells;

(ii) cultivating the transfected cells under conditions which permit expression of the nucleic acid molecules present in the vectors;

(iii) identifying those vector pools which lead after transfection to expression of said reporter gene in the mammalian cells;

(iv) optionally subdividing the vector pool(s) identified in step (iii) and repeating step (i) to (iii); and

(v) isolating from the so-identified vector pool(s) the nucleic acid molecule present in the vector(s) and testing its product for tumor suppressor activity.

This novel functional expression cloning technique relies on the transcriptional induction of a gene coding for a G-protein coupled receptor (GPCR) which in its activated form stimulates the cAMP signaling pathway which in turn results in the induction of cAMP responsive genes.

In the method of the invention said transcriptional induction of GPCR genes is conferred by the expression of a functional tumor suppressor, the presence of which is detected by subsequent activation of the endogenous signal transduction pathway and can be monitored by activation of a downstream amplificator, for example, a cAMP responsive reporter gene. The GPCR the expression of which is induced by the tumor suppressor may be any GPCR which is active under the culture conditions employed, in the sense that it activates the cAMP signaling

pathway. For example, the GPCR may be constitutively active or activated by a cognated ligand. Examples for GPCR which are positively coupled to cAMP production are the calcitonin, parathyroid hormone, thyrotropin, β-adregenic and pituitary adenylate cyclase activating peptide (PACAP) receptors. One may specifically target a tumor suppressor which induces the transcription of a certain GPCR by adding a ligand or a certain combination of different ligands which activate the cognate GPCR the expression of which is induced by the tumor suppressor. In a preferred embodiment the ligand is the peptide PACAP and the GCPR is the PACAP-type 1 (PVR1) receptor (Spengler et al., Nature 365 (1993), 170-175).

As mentioned above, the GPCR may not require to be activated by the addition of a ligand. These may be a, for example, naturally occurring constitutive active native or mutated GPCR.

Regulation of PVR1-receptor expression depends on activation of the endogenous gene by mechanisms at present not known. Delineation of this molecular pathway could allow to determine the cis-regulatory sequences in the PVR-1 receptor used for transactivation by TSGs. Therefore fusion of such a TSG-responsive region to a reporter gene could present an alternative usage of the present method.

Cells which are suitable for the purpose of the described method are such cells which reveal to elevations of intracellular cAMP a nuclear response leading to transcriptional activation of genes linked to a cAMP-responsive element.

Examples for such cells are those of cell line porcine renal epithelial LLC-PK1 (ATCC CC101) and human osteosarcoma Saos-2 (ATCC HTB 85). A suitable cell line is characterized by the presence of a cAMP dependent protein kinase A (PKA) and a cAMP response element (CRE)-binding protein which mediate the effects of cAMP. After binding of cAMP PKA is activated and able to phosphorylate the CRE-binding protein which is activated to turn on the transcription of cAMP responsive genes, namely genes which contain a short regulatory sequence called CRE which provide for binding of the CRE-binding

protein; for a general review of the cAMP signaling pathway; see, for example, Alberts et al., Molecular Biology of the Cell, 3rd ed. Garland Publ., Inc. N.Y. (1994) Chapter 15.

Other suitable cell lines may be identified by the person skilled in the art by screening a panel of cell lines for efficient expression of the expression vector employed combined with high transfection efficiency and with high responsiveness to cAMP. Expression and transfection efficiency may be optimized by conventional methods known in the art. Responsiveness to cAMP can be determined, for example, by transfection of a plasmid encoding and expressing a receptor which is positively coupled to cAMP production such as, for example, a GPCR as described above and measuring the induction of the cAMP mediated cellular response. The cAMP mediated cellular response may be determined by, for example, quantifying the production of cAMP or by monitoring the activation of an endogenous cAMP responsive gene and/or of a cotransfected cAMP responsive reporter gene. A detailed method for identifying a suitable cell line is described, for instance, in Example 1.

The cAMP-responsive element present in the regulatory elements which drive expression of the reporter gene on the first vector may in principle be any element known to respond to elevated levels of intracellular cAMP with an increase of transcription rate of a cis-linked sequence. Such cAMP-responsive elements are known, for example, from the genes encoding peptide hormones, for example somatostatin and corticotropin releasing hormone and are described in Spengler et al., Mol. Endocrinology 6 (1992), 1931-1941; Comb et al., Nature 323 (1986), 353-356; Roesler et al., J. Biol. Chem. 263 (1988), 9063-9066; Karin, Trends Genet. 5 (1989), 65-67 and Lalli and Sassone-Corsi, J. Biol. Chem. 269 (1994), 17359-17362. Preferably, the cAMP-responsive element has the nucleotide sequence of the consensus sequence of cAMP-responsive elements described in the literature. Most preferably, the cAMP-responsive element is one known from a human corticotropin releasing hormone gene which is, advantageously, flanked

by sequences naturally surrounding it. Advantageous is also the use of a cAMP-responsive element comprising the consensus sequence of an AP1 element or a degenerated version thereof.

The regulatory elements comprising the cAMP-responsive element(s) and which direct expression of the reporter gene in the transfected cells, may be any suitable elements capable of directing expression in the chosen cells. These elements normally comprise a promoter sequence, in particular a minimal promoter, preferably one which comprises

(a) a TATA or a CAAT box, preferably in conjunction with an Sp1-dependent activator, or

(b) an initiator element (Inr) in conjunction with an Sp1-dependent activator.

In a preferred embodiment the regulatory elements are derived from mammary mouse tumor virus (MMTV) promoter.

The reporter gene present in the first vector may be any suitable reporter gene the expression of which can be detected in the transfected cells. Preferably, a reporter gene is chosen the expression of which can be easily detected, for example, by photometric or fluorometric methods, isotopic labeling or by a staining reaction. Examples for reporter genes preferably used in the method according to the invention are those coding for chloramphenicol-acetyltransferase (CAT), ß-galactosidase (ß-Gal), secreted alkaline phosphatase (SEAP) or growth hormone (GH). Most preferably, a gene coding for luciferase is used.

The term "pool of expression vectors" in step (i) (b) of the method according to the invention is meant to be understood as a plurality of vector molecules which are either identical or not and which are adapted for expression in the transfected cells. Such vector molecules comprise regulatory elements which are capable of directing expression of a linked sequence in the transfected cells. Furthermore, these vector molecules comprise nucleic acid sequences linked to said regulatory elements which code for a gene product and which may either be identical or different in the members of the vector pool. The vectors of said vector pool may

furthermore comprise sequences which ensure replication in prokaryotic host cells as well as sequences which ensure replication in the transfected eukaryotic cells. Such a pool of expression vectors may be, for example, a cDNA library or a genomic library cloned in expression vectors suitable for expression in the transfected cells.
In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1(GIBCO BRL).

In a preferred embodiment the nucleic acid molecules present in the vectors of the vector pool are cDNA molecules. In particular, said cDNA may be prepared from RNA obtained from any organism or tissue, namely from any animal, bacterial, fungal or plant cells or from viruses. Most preferably, the RNA is obtained form mammalian cells. In such case the RNA is preferably derived from a specific tissue or organ of a mammal, e.g., intestine, stomach, lung, adrenal gland, kidney, mammary gland, prostate, testis, most preferably said tissue is pituitary gland, brain or ovary.

If a vector pool is identified in step (iii) of the method according to the invention then it is either possible to isolate from the original pool of the so-identified vector pool the nucleic acid molecules present in the vectors of the vector pool and characterize the encoded products or one can further subdivide the original vector pool, for example, if it consists of vectors with a plurality of different inserts, so as to reduce the number of different vectors per pool and repeat the method with the subdivisions of the original pool. Depending on the complexity of the pool this can be done for several times, preferably so often until the vector pool identified in step (iii) of the method only comprises a limited number of vectors which differ with respect to their inserted nucleic acid molecule. Normally the vector pool used in step (i) for transfecting the cells has previously been isolated from a mixture of bacteria harboring different vectors and which, thus, constitute a kind of library. Subdivision of the vector pool for the purpose of step (iii) of the method can

therefore be achieved by subdividing said library comprising the bacteria so that the diversity of the vectors with respect to the inserted nucleic acid sequences is lower in the subdivisions than in the original library. From these subdivisions of the library the expression vectors can then be isolated. These isolates then represent subdivisions of the original vector pool.

The nucleic acid molecules present in vectors of a vector pool identified by (iii) of the method according to the invention can be isolated from the vectors, e.g. by digestion with suitable restriction enzymes and can be further characterized, for example by restriction mapping, sequencing etc. The expression products of the thus obtained nucleic acid molecules are then tested for their tumor suppressor activity. This can be done, for example, by measuring the suppression of colony formation of transformed cell lines transfected with a TSG expressing vector. Furthermore, an inducible expression system may be employed to measure the suppression of growth of tumor cells which are stably transfected with an inducible TSG. After induction of TSG expression the growth of the tumor cells can be monitored and compared to non-TSG expressing tumor cells. An example of an inducible expression system is the tetracycline-regulated gene expression but others may be used as well, for example, heavy metal inducible expression systems. The suppressor activity of the putative TSG can also be detected by assaying its ability of suppressing anchorage-independent cell growth after induction of the TSG of the stably transfected tumor cells. Furthermore, the loss of tumorigenicity under expression of the TSG can be tested, for example, by implanting the tumor cells harboring an inducible TSG in nude mice and monitoring tumor development after induction of TSG expression. The capability of TSGs of recruiting apoptotic programs to inhibit growth of tumor cells is evidenced, for example, by the failure of the induced TSG expressing cells to convert MTT, their shrinking in size, their abundance in phase contrast microscopy, blebbing of their membrane, and rounding up of the cells before detaching from the plates. The cell death may also be accompanied by fragmentation of the DNA into a ladder of regular subunits. All the methods

r ferred to are well known in the art and are described in the Examples of the present application and/or are described in Zhou et al., Proc. Natl. Acad. Sci. USA 91 (1994), 4165-4169; Chen et al., Science 250 (1990), 1576-1580; Baker et al., Science 249 (1990), 912-915; Diller et al., Mol. Cell. Biol. 10 (1990), 5772-5781; Casey et al., Oncogene 6 (1991), 1791-1797; Cheng et al., Cancer Research (1992), 222-226; Wang and Prives, Nature 376 (1995), 88-91; Mercer et al., Proc. Natl. Acad. Sci. USA 87 (1990), 6166-6170; Antelman et al., Oncogene 10 (1995), 697-704; Gossen et al., Trends Biotech. 12 (1994), 58-62; Gossen and Bujard, Proc. Natl. Acad. Sci. USA 89 (1992), 5547-5551.

The expression products of the identified nucleic acid molecules can be furthermore characterized by expressing them in prokaryotic host cells and purifying them. Subsequently, enzymatic and/or other biological activities can be determined by in-vitro assays. Expression in eukaryotic host cells or in-vitro transcription and translation systems may furthermore provide information about possible phoshporylation and/or glycosylation patterns etc.

As demonstrated in the examples of the present invention, the above-described method is suitable to identify and isolate nucleic acid molecules which encode proteins having the biological activity of a tumor suppressor.

Thus, in another aspect the present invention relates to nucleic acid molecules obtainable by a method according to the invention which encode a protein or polypeptide having tumor suppressor activity. Examples for such nucleic acid molecules are those described above. In a preferred embodiment the nucleic acid molecules according to the invention are DNA molecules, most preferably cDNA molecules.

Nucleic acid molecules according to the invention can be derived from any organism, namely from animals, plants, fungi, bacteria or viruses. In a preferred embodiment the nucleic acid molecules according to the invention are derived from a mammal, most preferably form a human or a mouse.

With the help of nucleic acid molecules identified and isolated by the method according to the invention it is possible to isolate the same or related molecules from the same or different organisms, for example, by screening genomic or

cDNA libraries with the nucleic acid molecules isolated according to described method as a probe.

Thus, the present invention also relates to nucleic acid molecules which hybridize to a nucleic acid according to the invention as described above and which code for a protein having tumor suppressor activity. In accordance with the present invention, a further nucleic acid molecule encoding a protein having tumor suppressor activity was isolated using a nucleic acid molecule comprising the coding sequence of SEQ ID NO. 1 as a probe. The nucleotide sequence of said nucleic acid molecule is given in SEQ ID NO. 16 encoding a protein having the amino acid sequence as depicted in SEQ ID NO. 17.

The overall identity between the coding sequences of SEQ ID NO. 1 and SEQ ID NO. 16 was 74.6% at the nucleotide level and 68.5% at the amino acid level. As described in Example 9, several domains could be identified in the amino acid sequence of this protein (SEQ ID NO. 17), each having its counterpart in SEQ ID NO. 2. As described in Examples 12 to 15, the protein encoded by the nucleic acid sequence of SEQ ID NO. 16 displays identical properties compared to those of the protein encoded by the nucleotide sequence of SEQ ID NO. 1 described above and, therefore, constitutes a member of the new class of TSGs established by the provision of the aforedescribed nucleic acid molecules and proteins. Thus, in a preferred embodiment, the present invention relates to nucleic acid molecules, wherein the nucleic acid molecule comprises a nucleotide sequence encoding the amino acid sequence given in SEQ ID NO. 17 or comprises the nucleotide sequence given in SEQ ID NO. 16:

Furthermore, the present invention relates to nucleic acid molecules which hybridize to a nucleic acid molecule according to the invention as described above and which encode a mutated version of a polypeptide encoded by a nucleic acid molecule as described above which has lost its tumor suppressor activity.

Furthermore, the present invention relates to nucleic acid molecules which represent or comprise the complementary strand of any of the abov described nucleic acid molecules or a part thereof. Such a molecule may either be a desoxyribonucleic acid or a ribonucleic acid. Such molecules comprise, for example, antisense RNA. Nucleic acid molecules according to this preferred embodiment of the invention which are complementary to a nucleic acid molecule as described above may also be used for repression of expression of a TSG, for example due to an antisense or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-B1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a nucleic acid molecule of the invention. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460.

The present invention also relates to nucleic acids molecules of at least 15 nucleotides in length which specifically hybridize to any one of the aforementioned nucleic acid molecules or to a complementary strand thereof. Said nucleic acid molecules may be used, for example, as probes for the detection of a TSG according to the invention or its mRNA. In a preferred embodiment said nucleic acid molecules are labeled. Methods for the detection of nucleic acids are well known in the art, e.g., Southern and northern blotting, PCR, primer extension. In another preferred embodiment said nucleic acid molecules may be used for the suppression of TSG expression.

Furthermore, the present invention relates to a vector comprising a nucleic acid molecule according to the invention. Examples for such vectors are pUC18, pBR322, pBlueScript.

In a preferred embodiment the nucleic acid molecule present in the vector is operatively linked to regulatory elements permitting expression in prokaryotic or

eukaryotic host cells. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the *lac* or *trp* promoter in E. coli, and examples for regulatory elements permitting expression in eukaryotic host cells are the *AOX1* or *GAL1* promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the nucleic acid molecule.

The invention also relates to a host cell comprising a vector according to the invention. In this context, the host cell may be a bacterial, fungal, plant or animal cell. In a preferred embodiment the host cell is a mammalian cell.

In a further embodiment the invention relates to a method for the production of a polypeptide having the biological activity of a tumor suppressor comprising culturing a host cell as defined above under conditions allowing the expression of the polypeptide and recovering the produced polypeptide from the culture. Such methods are described, for example, in LaVallie and McCoy, Curr. Opin. Biotech. 6 (1995), 501-506; Wong, Curr. Opin. Biotech. 6 (1995), 517-522; Romanos, Curr. Opin. Biotech. 6 (1995), 527-533; Keränen and Penttilä, Curr. Opin. Biotech. 6 (1995), 534-537; Williams et al., Curr. Opin. Biotech. 6 (1995), 538-542; Davies, Curr. Opin. Biotech. 6 (1995), 543-547; Holmgren, Annu. Rev. Biochem. 54 (1985) 237-271 or LaVallie et al., Bio/Technology 11 (1993) 187-193.

Furthermore, the invention relates to a polypeptide encoded by a nucleic acid molecule according to the invention or produced by the above-described method, which has tumor suppressor activity.

In this context it is also understood that the polypeptides according to the invention may be further modified by conventional methods known in the art. By providing the polypeptides according to the present invention it is also possible to determine the portions relevant for their biological activity, namely their tumor suppressor activity. This may allow the construction of chimeric proteins comprising an amino acid sequence derived from a tumor suppressor protein of the invention which is crucial for tumor suppression and other functional amino acid sequences e.g. nuclear localization signals, transactivating domains, DNA-binding domains, hormone-binding domains, protein tags (GST, GFP, h-myc peptide, Flag, HA peptide) which may be derived from the same or from heterologous proteins.

The present invention also relates to a polypeptide encoded by a nucleic acid molecule according to the invention or produced by the above-described method, which is a mutated version of an above-described polypeptide which has lost its tumor suppressor activity.

The present invention furthermore relates to antibodies specifically recognizing a polypeptide according to the invention which has a tumor suppressor activity. Namely, the invention relates to antibodies which specifically recognize polypeptides according to the invention irrespective of whether they are functional tumor suppressors or whether they are mutated forms which have lost their tumor suppressor activity.

In a preferred embodiment the antibody specifically recognizes a polypeptide according to the invention which has tumor suppressor activity but does not recognize a polypeptide which is a mutated version of such a polypeptide and which has lost its tumor suppressor activity.

In another preferred embodiment the antibody specifically recognizes the mutated form which has lost its tumor suppressor activity but not the corresponding polypeptide having tumor suppressor activity.

In a preferred embodiment said antibody is a monoclonal antibody.

Anti-tumor suppressor protein antibodies can be prepared by well known methods using a purified tumor suppressor protein according to the invention or a synthetic fragment derived therefrom as an antigen. Monoclonal antibodies can be prepared, for example, by the techniques as described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. The antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Furthermore, antibodies or fragments thereof to the aforementioned tumor suppressor proteins can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. These antibodies can be used, for example, for the immunoprecipitation and immunolocalization of the proteins of the invention as well as for the monitoring of the presence of such proteins, for example, in recombinant organisms, and for the identification of compounds interacting with the proteins according to the invention. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies which bind to an epitope of the protein of the invention (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13).

Moreover, the present invention relates to a pharmaceutical composition comprising at least one of the aforementioned nucleic acid molecules, vectors, polypeptides and/or antibodies according to the invention either alone or in combination, and optionally a pharmaceutically acceptable carrier or exipient.
Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by conventional methods. The pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different

ways, e.g. by intravenous, intraperetoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{22} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

The pharmaceutical compositions according to the invention can be used for the prevention or treatment of different kinds of diseases, for example, cancer, namely benign or malignant tumors, of acquired or inborn neuronal disorders, neurodegenerations and related disorders. With respect to the prevention or treatment of tumors, said tumors are preferably derived from endocrine or neuronal tissues, i.e. intestine, stomach, lung, adrenal gland, kidney, mammary gland, prostate, testis, most preferably said tissue is colon, pancreas, thyroid, pituitary gland, brain, breast or ovary.

In a first aspect it is possible to use a pharmaceutical composition which comprises a nucleotide sequence which encodes a non-mutated form of a protein having tumor suppressor activity for gene th rapy. As described above tumors or other diseases often evolve when cells lose both functional copies of a tumor suppressor gene. In such a case introduction of functional copies of the

corresponding g ne may help to ameliorate the situation. For example, research pertaining to gene transfer into cells of the nervous system is one of the fastest growing fields in neuroscience. Gene therapy, which is based on introducing therapeutic genes into cells of the nervous system by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Gene therapy of the nervous system could be applied for three general purposes: genetic diseases, acquired degenerative encephalopathies, and control of malignant neoplasia.

In genetic diseases the introduction of a normal or a functionally adequate allele of a mutated nuclear gene represents gene-replacement therapy, which is applicable mainly to monogenetic recessive disorders.

In the field of neurobiology, for example, it has long been recognized that the balance between cellular proliferation and cell death during embryogenesis is a key factor in formation of the central nervous system (CNS). The recent definition of molecular mechanisms that drive the cell-division cycle and programmed cell dead provides an opportunity to investigate the molecular interactions that co-ordinate cell-cycle regulation with CNS-pattern formation, neural differentiation and histiogenesis. It appears evident that not only is the cell-division cycle regulated by developmentally controlled molecular signals to halt or proceed, but gene products that drive the cycle can also influence the course of neural differentiation and apoptosis. The neurotrophic strategy for the regulation of neuronal numbers may be only one example of a general mechanism that help to regulate the numbers of many other vertebrate cell types, which also require signals from other cells to survive. These survival signals seem to act by suppressing an intrinsic cell suicide program, the protein components of which are apparently expressed constitutively in most cell types. TSGs have emerged during the last years as major players in this area. Mice deficient for Rb revealed massive neuronal cell death due to the failure to stop cell division. A subset of p53-deficient mice (8 to 16%) exhibit exencephaly and a large population (40%) of Brca1-deficient mice embryos suffered to varying degrees of spina bifida and anencephalopathy. In addition, these animals displayed a disorganisation of the

neuroepithelium with signs of rapid proliferation and excessive cell death. Thus, it appears as if TSGs are intimately involved in CNS formation and that the balance between growth-constraining elements and neurotrophic support is a key event in formation of neuronal architecture. In this respect, nucleic acid molecules according to the invention which code for proteins with tumor suppressor activity are potential candidates to participate in these processes. Importantly, the nucleotide sequences of SEQ ID NO. 1 and SEQ ID NO. 16 which encode novel tumor suppressors hybridize to total RNA isolated from different brain areas of the mature brain. This opens the perspective that the subtle balance between promoters of apoptosis like the protein encoded by SEQ ID NO. 1 or SEQ ID NO. 16 and protectors like neurotrophins safeguard functional integrity of the mature brain. An increasing list of neurodegenerative disorders including Alzheimer disease and Chorea Huntington have been reported to reveal increased incidence of apoptotic cell death. In this view gene targeting of nucleic acid molecules coding for a protein having the amino acid sequence as depicted in SEQ ID NO. 2 or SEQ ID NO. 16 or nucleic acid molecules coding for related proteins of the invention bears the potential promise to mitigate apoptotic cell death under various circumstances and to increase sensitivity to neurotrophic treatments aimed to preserve neuronal cell number and neuronal viability.

Furthermore, recent reports indicated that p53-dependent apoptosis modulates the cytotoxic effects of common antitumor agents such as ionizing radiation, fluorouracil, etoposide, and doxrubicin. Cells lacking wild-type p53 are resistant to these agents, whereas cells expressing wild-type p53 are sensitive to them and undergo cell death by apoptosis. These observations raise the exciting prospect that p53 mutations may provide a genetic basis for drug resistance. In the presence of p53, oncogene-expressing cells can form tumors, but cell survival is limited by their increased susceptibility to apoptosis. Conversely, p53 loss contributes directly to immortalization and tumorgenesis, probably by abrogating an intrinsic senescence program. As a consequence, selection against p53 often occurs late in tumor progression. Anticancer agents may simply activate the apoptotic program intrinsic to these sensitized cells. These observations predict

that reintroduction of normal tumor suppressor function into tumors harboring mutations in tumor suppressor genes will enhance apoptosis after radiation or chemotherapy, an approach that has proved successful for cisplatin in a lung carcinoma cell line.

Since it was observed that introduction of a nucleic acid molecule according to the invention caused apoptotic cell death in transformed cell lines, which in part exceeded the one caused by p53, these novel TSGs present a powerful option of high potential interest in gene therapy experiments. Though p53 and the protein encoded by SEQ ID NO. 1 or SEQ ID NO. 16 induce at a descriptive level the same responses, namely cell-cycle regulation and cell death, the underlying molecular routes diverge. This observation originates from the fact that the DNA-binding domain of the protein encoded by SEQ ID NO. 1 and SEQ ID NO. 16 is organized in a typical zinc finger structure, which is unrelated to the central DNA-binding domain of p53. Therefore, the protein encoded by SEQ ID NO. 1 and SEQ ID NO. 16 and related proteins could replace p53 in gene therapy strategies. Importantly p53 seems only to trigger growth arrest and not cell death in some cell types and under some conditions. In line with this view we demonstrated that restoration of inducible p53 function in the p53-negative cell line Saos-2 (human, osteosarcoma) installed preferentially a growth and a comparatively weak apoptotic response, whereas Saos-2 cells became highly apoptosis proficient under expression of the protein encoded by SEQ ID NO. 1 and SEQ ID NO: 16. This differential apoptotic response emphasizes the idea that this protein and other TSGs of the invention and p53 supply different molecular routes to apoptosis and open the exciting perspective that apoptosis competency is a tissue-specific encoded genetic program. Conclusively tissue-specific TSGs as those provided by the present invention could encode specific properties to guide tumorigenic cells to apoptotic cell death and their potency to do so could surpass p53 as illustrated for the proteins encoded by SEQ ID NO. 1 and SEQ ID NO. 16 in Saos-2 cells.

Importantly again, the understanding of p53 function as an example for a tumor suppressor gene suggest a basis for the association between p53 mutations and

poor patient prognosis. Thus, p53 mutations, which are with 50% among the most common alterations observed in human cancer, may be a significant impediment to successful cancer therapy. For example, p53 mutations dramatically reduce the probability that patients with B cell chronic lymphocyte leukemia will enter remission after chemotherapy. Similarly evaluation of the status of proteins encoded by nucleic acid molecules according to the invention and related proteins in tumor samples could serve as an decisive parameter for the extent and necessity of surgical resection and the need for adjuvant therapy. In a more general view, the status of nucleic acid molecules according to the invention encoding proteins with the capability to induce apoptosis could become a decisive criteria to develop treatment priorities for individual tumor specisms. In another important aspect the above-mentioned pharmaceutical compositions may be used in immuntherapy. The well-characterized mutations of a TSG also suggest the possibility of immuntherapy or even a cancer vaccine, which would alert the body's immune system to the mutant forms of the protein. Cross-reactivity to wild-type forms has to be considered as a potential unwanted side-effect with profound implications since abating wild-type function through uncontrolled autoimmunoreactivity would dramatically enhance the risk of additional tumor formation. In this regard, it is advantageous to use tissue specific TSGs, such as the one represented in SEQ ID NO. 1 or in SEQ ID NO: 16, since in this way the risk of the above-mentioned unwanted side-effect can be substantially lowered. Vectors comprising a nucleic acid molecule of the invention may be stably integrated into the genome of the cell or may be maintained in a form extrachromosomally, see, e.g., Calos, Trends Genet. 12 (1996), 463-466. On the other hand, viral vectors described in the prior art may be used for transfecting certain cells, tissues or organs. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others. Delivery of nucleic acid molecules to a specific site in the body for gene therapy may also be accomplished using a biolistic delivery system,

such as that described by Williams (Proc. Natl. Acad. Sci. USA 88 (1991), 2726-2729).

Standard methods for transfecting cells with nucleic acid molecules are well known to those skilled in the art of molecular biology, see, e.g., WO 94/29469. Gene therapy to prevent or decrease the development of tumors may be carried out by directly administering the nucleic acid molecule of the invention encoding a TSG to a patient or by transfecting cells with said nucleic acid molecule of the invention *ex vivo* and infusing the transfected cells into the patient. Furthermore, research pertaining to gene transfer into cells of the germ line is one of the fastest growing fields in reproductive biology. Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., WO94/29469, WO 97/00957 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein. The nucleic acid molecules comprised in the pharmaceutical composition of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) containing said nucleic acid molecule into the cell. Preferably, said cell is a germ line cell, embryonic cell, or egg cell or derived therefrom if the production of transgenic non-human animals is envisaged.

It is to be understood that the introduced nucleic acid molecules encoding the protein having the biological activity of a tumor suppressor express said protein or activator after introduction into said cell and preferably remain in this status during the lifetime of said cell. For example, cell lines which stably express said protein having the biological activity of a tumor suppressor may be engineered according to methods well known to those skilled in the art. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the recombinant DNA molecule or vector of the invention and a selectable marker, either on the same or separate vectors. Following the introduction of

foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows for the selection of cells having stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the protein having the biological activity of a tumor suppressor.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, Cell 11(1977), 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska, Proc. Natl. Acad. Sci. USA 48 (1962), 2026), and adenine phosphoribosyltransferase (Lowy, Cell 22 (1980), 817) in tk, hgprt or aprt cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, Proc. Natl. Acad. Sci. USA 77 (1980), 3567; O'Hare, Proc. Natl. Acad. Sci. USA 78 (1981), 1527), gpt, which confers resistance to mycophenolic acid (Mulligan, Proc. Natl. Acad. Sci. USA 78 (1981), 2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, J. Mol. Biol. 150 (1981), 1); hygro, which confers resistance to hygromycin (Santerre, Gene 30 (1984), 147); or puromycin (pat, puromycin N-acetyl transferase). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

In another aspect it is possible that the pharmaceutical compositions comprise the functional proteins encoded by the nucleic acid molecules according to the invention or proteins which repres nt mutated versions of th se proteins which occur in various diseases. These compositions may either be useful to restore

normal tumor suppressor activity in cells which have lost both functional copies of the relevant gene or for immuntherapy as already described above.

Furthermore, the use of pharmaceutical compositions which comprise antisense-oligonucleotides which specifically hybridize to RNA encoding mutated versions of a tumor suppressor according to the invention or which comprise antibodies specifically recognizing such mutated versions but not the functional wild-type form is conceivable in cases in which the concentration of the mutated form in the cells should be reduced. The pharmaceutical compositions according to the invention can be used for the treatment of various kinds of diseases. Thus, the present invention also relates to methods for the treatment or prevention of tumors or neuronal disorders or for the delay of the reoccurrence of tumors or neuronal disorders which comprises the administration of an effective dose of a pharmaceutical composition according to the invention to the subject.

Furthermore, any of the aforementioned nucleic acid molecules, vectors, polypeptides and/or antibodies according to the invention either alone or in combination can be used for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject. Preferably, said disease is a tumor or a neuronal disorder, for example, a tumor or a neuronal disorder as described above.

The invention also relates to a diagnostic composition comprising at least one of the aforementioned nucleic acid molecules, vectors, polypeptides and/or antibodies according to the invention either alone or in combination, and optionally suitable means for detection.
Said diagnostic compositions may be used for methods for detecting expression of a tumor suppressor by detecting the presence of mRNA coding for a tumor suppressor which comprises obtaining mRNA from a cell and contacting the mRNA so obtained with a probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a nucleic acid molecule

encoding a tumor suppressor under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and ther by detecting the expression of the tumor suppressor by the cell.

Furthermore, the invention comprises methods of detecting the presence of a tumor suppressor of the present invention in a sample, for example, a cell sample, which comprises obtaining a cell sample from the subject, contacting said sample with one of the aforementioned antibodies under conditions permitting binding of the antibody to the tumor suppressor, detecting the presence of the antibody so bound, for example, using immuno assay techniques, for example, radioimmunoassay or enzymeimmunoassay. Furthermore, one may specifically detect and distinguish polypeptides which are functional tumor suppressors from mutated forms which have lost or altered their tumor suppressor activity by using an antibody which either specifically recognizes a polypeptide which has tumor suppressor activity but does not recognize an inactive form thereof or which specifically recognizes an inactive form but not the corresponding polypeptide having tumor suppressor activity. The antibodies of the present invention may also be used in affinity chromatography for purifying the polypeptides of the present invention and isolating them from various sources.

The invention also relates to a method for diagnosing in a subject a predisposition to a tumor or a disorder associated with the expression of a tumor suppressor allele which comprises isolating DNA from victims of the tumor or the disorder associated with the expression of a tumor suppressor; digesting the isolated DNA with at least one restriction enzyme; electrophoretically separating the resulting DNA fragments on a sizing gel; contacting the resulting gel with a nucleic acid probe as described above capable of specifically hybridizing to DNA encoding a tumor suppressor and labeled with a detectable marker; detecting labeled bands on the gel which have hybridized to the labeled probe to create a band pattern specific to the DNA of victims of the tumor or the disorder associated with the expression of a tumor suppressor; preparing the subject's DNA according to the above-mentioned steps to produce detectable labeled bands on a gel; and comparing the band pattern specific to the DNA of victims of the tumor or the

disorder associated with the expression of a tumor suppressor and the subject's DNA to determine whether the patterns are the same or different and to diagnose thereby predisposition to the tumor or the disorder if the patterns are the same. The detectable markers of the present invention may be labeled with commonly employed radioactive labels, such as, for example, ^{32}P and ^{35}S, although other labels such as biotin or mercury may be employed as well. Various methods well-known to the person skilled in the art may be used to label the detectable markers. For example, DNA sequences and RNA sequences may be labeled with ^{32}P or ^{35}S using the random primer method. Once a suitable detectable marker has been obtained, various methods well-known to the person skilled in the art may be employed for contacting the detectable marker with the sample of interest. For example, DNA-DNA, RNA-RNA and DNA-RNA hybridizations may be performed using standard procedures. Various methods for the detection of nucleic acids are well-known in the art, e.g., Southern and northern blotting, PCR, primer extension and the like. Furthermore, the mRNA, cRNA, cDNA or genomic DNA obtained from the subject may be sequenced to identify mutations which may be characteristic fingerprints of TSG mutations in tumors or disorders associated with the expression of TSG or mutated versions thereof. The present invention further comprises methods wherein such a fingerprint may be generated by RFLPs of DNA or RNA obtained from the subject, optionally the DNA or RNA may be amplified prior to analysis, the methods of which are well known in the art. RNA fingerprints may be performed by, for example, digesting an RNA sample obtained from the subject with a suitable RNA-Enzyme, for example RNase T_1, RNase T_2 or the like or a ribozyme and, for example, electrophoretically separating and detecting the RNA fragments as described above.

It is furthermore possible to use the TSGs and proteins according to the invention for the design of "killer genes " (Da Costa et al., Proc. Natl. Acad. Sci. USA 93 (1996), 4192-4196). It has become clear that tumorigenesis is driven by alterations in genes that control cell growth and cell death. Gene therapy could be aimed at specifically kill tumor cells expressing mutated forms of tumor

suppressor genes. In outline, the target protein, i.e. the mutated tumor suppressor, binds to exogenously introduced gene products, resulting in transcriptional activation of a toxic gene. This strategy may be generally applicable to neoplastic disease in which the underlying patterns of genetic alterations or abnormal gene expression are known (Da Costa et al., Proc. Natl. Acad. Sci. USA 93 (1996), 4192-4196).

Conceivable is also the restoration of the wild-type conformation of mutated tumor suppressor proteins.

Some genetic changes lead to altered protein conformational states. For example, mutant p53 proteins possess a tertiary structure that renders them far less capable of binding to their wild-type DNA recognition elements. Restoring the normal or regulated conformation of mutated proteins is the most elegant and specific means to correct these molecular defects, although it is difficult. Of particular interest in this regard is the zinc finger structure of the protein encoded by SEQ ID NO. 1 or SEQ ID NO. 16 if the DNA-binding potency is reduced in mutated proteins. The fact that the nucleic acid molecules having the nucleotide sequence as depicted in SEQ ID NO. 1 or SEQ ID NO. 16 are expressed in a tissue-specific manner deserves particular attention. All pharmacological manipulations aimed at restoration of wild-type conformation p53, bear the risk to interfere with the wild-type function of this tumor suppressor in neighboring non-tumorgenic tissues with profound side-effects. In contrast the targeting of tissue-specific TSGs could remarkably extend the applicability of a targeting approach, since considerable higher concentrations of the molecules and/or long-lasting derivatives can be employed at a lowered risks for demetrial side-effects.

Thus, the nucleic acid molecules and encoded proteins of the present invention may also be used to design and/or identify molecules and compounds which are capable of activating or inhibiting the wild-type function of a tumor suppressor. These molecules and compounds may be small organic compounds, antibodies, petidomimetics, PNAs or peptides (Milner, Nature Medicine 1 (1995), 879-880; Hupp et al., Cell 83 (1995), 237-245; Gibbs and Oliff, Cell 79 (1994), 193-198).

Thus, the present invention further relates to a process for identifying compounds effective as antagonists or agonists to a tumor suppressor comprising:

(a) contacting a cell which expresses the polypeptide of the invention with a compound to be screened; and

(b) determining if the compound inhibits or enhances activation of the tumor suppressor.

Said compounds may be comprised in, for example, samples, cell extracts from, e.g. plants, animals or microorganisms. Furthermore, said compounds may be known in the art but hitherto not known to be an antagonist/inhibitor or agonist/activator of the protein of the invention. Preferably said sample comprises substances of similar chemical and/or physical properties, most preferably said substances are identical. The compounds which can be prepared and identified according to a use of the present invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, ligands, hormones, peptidomimetics, PNAs or the like. The identification of compounds which are capable of activating or inhibiting the wild-type function of a tumor suppressor can be performed according to the methods known in the art, for example as described in EP-A-0 403 506. The antagonists identified according to the above-described method may reveal new classes of substances involved in, e.g., tumorigenesis. The agonist identified according to the method of the invention may prove useful for therapy of tumorous diseases. Hence, the present invention also relates to antagonists/inhibitors and agonists/activators to the polypeptide of the invention or obtainable according to the method described above.

These and other embodiments are disclosed or are obvious from and encompassed by the description and examples of the present invention. For example, further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on Internet, e.g.

under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The pharmaceutical compositions, uses, methods of the invention can be used for the treatment of all kinds of diseases hitherto unknown as being related to or dependent on the modulation of tumor suppressor genes or their encoded protein. The pharmaceutical compositions, methods and uses of the present invention may be desirably employed in humans, although animal treatment is also encompassed by the methods and uses described herein.

The Figures show:

Figure 1: Bop1 Sequence and Tissue Distribution

(A) Sequence of Bop1 protein. Cysteine and histidine residues of the seven zinc finger motifs of the C_2H_2 type are boxed. A putative phosphorylation site for cyclin-dependent kinases (Cdks) corresponding to the consensus motif (b/p)(S/T)Pxb located at residues 56-60 is underlined. A putative phosphorylation site for protein kinase A (PKA) at residue 666 is indicated (*).

(B) Schematic representation of Bop1 clones. Clone p2195 and p1270 were derived from the AtT-20 corticotroph tumor cell line. Clone B-16 was isolated from a BALB/c pituitary library and encodes the same protein identified in p2195 and p1270. The coding region of p1270 and B-16 is interrupted at residue 658 by a 630 bp insertion. The sequences at the boundaries of this insertion are displayed in the lower part of the figure and are in excellent agreement with consensus

exon-intron junctions and preserve the reading frame. Restriction sites for EcoR I (R), BamH I (B) and Not I (N) are indicated.

(C) Expression of Bop1 mRNA in mouse tissue. Bop1 distribution was assessed by northern blot analysis of total RNA prepared from different brain regions (olfactory bulb (Olf), frontal cortex (fCx), occipital cortex (oCx), hippocampus (Hip), hypothalamus-thalamus (HyT), brain stem (BSt), cerebellum (Crb) and peripheral tissues (anterior pituitary gland (Pit)), heart (Hea), liver (Liv), stomach (Sto), intestine (Int), kidney (Kid), adrenal gland (Adr), spleen (Spl), lung (Lun)). Ethidium bromide staining of the gel is shown in the insert to document equal and intact amounts of each RNA preparation.

Figure 2: Bop1 and p53 Alter Proliferation of LLC-PK1 and Saos-2 Cells

Anhydrotetracycline(ATc)-regulated expression of Bop1 and p53 was established in LLC-PK1 and Saos-2 cells.

(A) Cell counts of the parent tTA clones (L-tTA and S-tTA) in comparison to Bop1- and p53-expressing LLC-PK1 (L-Bop and L-p53, respectively) and Saos-2 (S-Bop and S-p53, respectively) clones in the presence (+) and absence (-) of ATc.

(B) Bop1 and p53 inhibit DNA-synthesis (BrdU) and cell viability (MTT). For each time point, BrdU incorporation or formazan blue formation were measured in the absence (-) or the presence (+) of ATc.

(C) Growth inhibition by Bop1 and p53 is serum independent. Cells were grown in the presence of the indicated amount of fetal bovine serum (10% or 0.1%) and in the presence (+) or absence (-) of ATc.

(D) Growth inhibition by Bop1 and p53 is reversible. Cells were seeded in Atc-containing medium, grown in the absence of ATc for 2 days before medium was renewed (arrowhead) with medium containing (-/+) or lacking (-/-) ATc.

Figure 3: Bop1 and p53 Inhibit Soft Agar Colony F rmation

Bop1 (L-Bop and S-Bop) and p53 (L-p53 and S-p53) clones were grown in the presence of ATc before plating into soft agar at densities of $1x10^5$ (No. 1+4), $5x10^4$ (No. 2+5) and $2.5x10^4$ (No. 3+6) cells per well in six-well plates. The repressor ATc was included in the upper row (+) and was omitted in the lower row (-). For photography on day 10, the soft agar was overlaid with MTT for 4 hr. Pictures shown are representative of three to five independent experiments.

Figure 4: Bop1 and p53 Induce Apoptotic Cell Death

(A) DNA laddering. Genomic DNA was isolated from Bop1 (L-Bop and S-Bop) and p53 (L-p53 and S-p53) expressing clones grown in the presence (+) or absence (-) of ATc for 3 days, centrifugated and soluble DNA was subjected to agarose gel electrophoresis and stained with ethidium bromide.

(B) Fluorescence microscopy of Bop1 and p53 clones stained with ethidium bromide and acridine orange. Cells (a: L-Bop; b: L-p53; c:S-Bop; d: S-p53) were grown in the absence of ATc for 3 days. Floating cells were collected, incubated with ethidium bromide and examined by fluorescence microscopy (510-550nm; x1000).

(C) DNA end labeling. S-Bop (Bop1) and S-p53 (p53) cells were grown for 3 days in the presence (black) or absence (grey) of ATc. Permeabilized cells were subjected to terminal transferase end labeling (TUNEL) in the presence of digoxigenin-labeled dUTP. Cells were then incubated with fluorescein-conjugated antidigoxigenin antiserum and subjected to flow cytometry.

Figure 5: Bop1 and p53 Regulate Cell Cycle Distribution

(A) Induction of G1 arrest by Bop1 and G2/M arrest by p53. S-Bop (upper panels) and S-p53 (lower panels) were grown in the presence (left) or absence (right) of ATc for 3 days. Propidium iodide-stained cells were analyzed by flow cytometry to

determine DNA content. Bop1 reduced the proportion of S-populations in S phase and G2/M phase from 37.8% and 17.5% to 24.5% and 12.6 %, respectively and increased cell population in G1 from 44.7% for the repressed state to 63.0% for the expressed state of S-Bop. For p53 a decrease in G1 and S phase from 39.4% to 31.8% and from 43.7% to 35.0% was observed, which was followed by a clear increase in G2/M from 16.9% to 33.2%.

(B) G1-Arrest by Bop1 is independent of p21^{Waf1} expression. S-tTA (tTA), S-p53 (p53) and S-Bop(Bop1) cells were grown in the presence (+) or absence (-) of ATc for 3 days. Western blots of total cell lysates were performed with anti-p21, anti-p53 and anti-GST-Bop1ΔZF antisera.

(C) Apoptotic cell death following Bop1 and p53 expression is unrelated to the cell cycle. TUNEL was carried out on permeabilized S-Bop (Bop1, upper panels) and S-p53 (p53, lower panels) cells grown in the presence (left) or absence (right) of ATc for 3 days. Subsequent staining with propidium iodide allowed simultaneous assessment of DNA content and apoptosis by flow cytometry. Grey dots in the boxed area represent cells with high TUNEL fluorescence and hence apoptotic. Dots in different shades of grey outside the boxed area correspond to living cells in G1 (bottom), S and G2/M (top) phase of the cell cycle. Apoptotic fluorescence threshold was set so that less than 5 % of S-tTA cells grown in the presence or absence of ATc were apoptotic. Apoptotic cells in the presence of ATc represent less than 5% of the cells in the case of S-Bop and less than 1% for S-p53. In the absence of ATc, 70% of S-Bop (65% of S-p53 resp.) cells displayed enhanced or high TUNEL fluorescence.

Figure 6: Transfer of PVR1 Gene Regulation through Bop1 Zinc Finger Domain and Nuclear Localization of Bop1

(A) Schematic representation of Bop1/steroid receptor hybrids. Abbreviations used are G and M for human glucocorticoid (GR) and mineralocorticoid receptor (MR) domains, respectively. The transactivation domain of the GR is represented by a hatched box, the MR hormone binding domain by a black box, and the MR

DNA-binding domain by grey box with the two zinc fingers indicated by vertical lines. The numbers above each box indicate amino acids.

(B) The zinc finger domain of Bop1 confers regulation of the PVR1 gene. Native Bop1 and p53 (left) or the hybrid GB_ZM (right) cDNAs were co-transfected with the cAMP-responsive reporter pΔMC16LUC into LLC-PK1 cells ($2x10^6$) and plated with aldosterone (Aldo; 10^{-9}M) or spironolactone (Spiro; 10^{-7}M). PACAP-38 (10^{-9}M) was added the next day for 4hr before harvesting the cells. To calculate induction ratios, luciferase activity was standardized on MTT values.

(C) Regulation of PVR1 by Bop1 requires transactivation (left). The construct ΔB_ZM is truncated for the GR transactivation domain and was tested under the same conditions as described above. Cytoplasmatic trapping of Bop1 prevents transactivation of the PVR1 gene (right). The native Bop1 cDNA was fused to the hormone-binding domain of the MR to create Bop_XM. Transfected LLC-PK1 cells ($2x10^6$) were replated in charcoal-treated serum and aldosterone or spironolactone were added separately. PACAP-38 (10^{-9}M) was added next day for 4hr before cells were harvested. To calculate induction ratios luciferase activity was standardized with MTT values.

(D) Bop1 is a nuclear protein. S-Bop cells were grown in the presence or absence of ATc for three days and simultaneously immunostained with rhodamine-conjugated phalloidin to stain actin filaments and with a rabbit antiserum raised against a GST-Bop1ΔZF fusion protein. The grey bar represents 25 μm.

Figure 7: Sequences of the ZAC/LOT family

(A) Sequence alignments of the ZAC/LOT proteins. Human ZAC (hZAC, identical to hLOT1), mouse ZAC (mZAC) and rat LOT1 (rLOT) were aligned according to a Clustal method. Residues that matched hZAC with one distance unit are boxed.

(B) Schematic repr sentation of the ZAC/LOT proteins structures. Each domain is indicated as a box: the 7 ZF of C2H2 type, the linker region, the Pro-repeats present in mZAC only, the Pro, Gln and Glu rich region, the Glu-clusters absent in

hZAC, and the C-terminus. The numbering of the aminoacid residues is indicated below the boxes.

(C) Phylogenetic tree for ZAC/LOT family. Only the ZF domains were taken into account for the analysis.

Figure 8: Localizations of hZAC and mZAC genes

(A) Chromosomal localizations. FISH with hZAC probe revealed spots on human chromosome 6 band 6q25; FISH with mZAC probe revealed spots on mouse chromosome 10 band $10A_2$ and FISH with mZAC probe revealed spots on human chromosome 6 band 6q25.

(B) Southern blots of human and mouse genomic DNA. Human (H) and mouse (M) genomic DNAs were digested with the indicated restriction enzymes, fractionated on a 1% agarose gel and blotted. The blot was first incubated with a radioactive mZAC probe and autoradiographed. Then, the membrane was stripped, the removal of the mZAC probe was checked, and the blot was hybridized with a hZAC probe and autoradiographed. The same data were obtained with another set of enzymes including Nco I, Sac II, Sac I, and Pst I.

Figure 9: Human tissue distribution of hZAC

A human RNA Master blot was hybridized with a hZAC probe and the signal intensity for each dot was measured using a storage phosphor imaging system (Bio-Rad).

There was also a weak expression of hZAC in adult peripheric leucocytes, spinal cord, liver, skeletal muscle and whole brain (less than 150 units). hZAC was expressed in different brain areas: mainly in the occipital lobe, thalamus and cerebral cortex (100-150 units), in other areas (amygdala, caudate, cerebellum, frontal lobe, hippocampus, medulla oblongata, putamen, substantia nigra, temporal lobe, subthalamic nuclei) the signals were very weak (<100 units).

Figure 10: hZAC is a nuclear transcriptional activator

(A) Nuclear localization of hZAC. Immunocytochemistry of SaOs-2 cells transfected with HA-tagged-hZAC (1µg plasmid) or -mZAC (200 ng plasmid) was performed using an anti-HA antibody and a FITC-conjugated secondary antibody. As cells were transiently transfected, positive transfected cells (indicated with an arrow) as well as negative non transfected cells were present on the same sample.

(B) Transcriptional activity of hZAC. SaOs-2 cells were transfected with plasmids encoding a fusion protein between the GAL4 DNA binding domain and either hZAC, mZAC, or the transactivation domains of SP1 or CTF, together with a luciferase reporter gene under the control of a GAL4 sensitive minimal promoter. Luciferase activity for each condition is indicated as a fold stimulation over basal.

Figure 11: hZAC inhibits colony formation

SaOs-2 cells were transfected with the plasmid encoding the puromycine resistance alone (pRK5-PUR), or together with plasmids encoding hZAC, mZAC and p53 in their sense and antisense (as) orientations. Puromycine was added for 9d and the resistant colonies were counted. Mock-transfected SaOs-2 cells were killed by puromycine. This experiment is representative of three independent experiments.

Figure 12: hZAC induces a G1 arrest

(A) Cell cycle distribution. SaOs-2 cells were cotransfected with pRK5 encoding CD20 and different amounts of pRK5 encoding either mZAC, hZAC or p53. CD20 positive and propidium iodide stained cells were analysed by flow cytometry to measure DNA content. This experiment is representative of 3 independent experiments.

(B) Western blots. SaOs-2 cells were transfected with different amounts of pRK5 encoding mZAC, hZAC or p53, as indicated. Western blot of total cell lysates was performed with anti-HA antibody.

Figure 13: hZAC induces apoptotic cell death

DNA laddering: SaOs-2 cells were transfected with different quantities of pRK5 plasmid (1500 ng, lane 1) or encoding CAT (1500 ng, lane 2), mZAC (50, 150 and 500 ng, for lanes 3, 4, 5, respectively), hZAC (500, 1000, 1500 ng, for lanes 6, 7, 8, respectively) or p53 (100ng, lane 9). This experiment is representative of 3 separate ones.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

Example 1: Cloning, structural analysis and tissue distribution of the TSG Bop 1

In order to isolate DNAs coding for different receptors positively coupled to adenylyl cyclase, we used a recently described expression cloning method (Spengler et al., Nature 365 (1993), 170-175). This method is based on transcriptional induction of a cAMP-responsive luciferase reporter gene by stimulation of adenylyl cyclase through activated target receptors.

Pools of clones from a mouse corticotroph pituitary tumor cell line (AtT-20) (Spengler et al., Nature 365 (1993), 170-175) cDNA library and from a new-born rat colliculi library were co-transfected with a cAMP-responsive reporter into LLC-PK1 cells according to the functional expression transductory cloning technique (*FETCH*).

This expression cloning technique r lies on the co-transfection of pools of clon s from a cDNA-expression library with a cAMP-responsive reporter into a mammalian cell line, most preferably LLC-PK1 cells.

In a previous series of studies we noted that a cAMP-responsive element derived from the hCRH-gene promoter conferred regulation by cAMP to heterologous promoters (Spengler et al., Mol. Endocrinology 6 (1992), 1931-1941). Further experiments demonstrated, that basal and induced expression depended strictly on the promoter context and the cell line employed. In this view a modified mammary mouse tumor virus promoter (ΔMTV) proved to be exceptional in combining a low level of basal expression with strong induction ratios in various cell lines tested including CV-1 (monkey kidney fibroblast), JAR (human choriocharcinoma), SK-N-MC (human neuroblastoma) and AtT-20 (mouse anterior pituitary) (Spengler et al., Mol. Endocrinology 6 (1992), 1931-1941). Properties of this reporter were further improved by increase of the number of CREs. These modifications allowed a synergistic enhancement in the response to cAMP approaching an induction plateau at a critical number of eight 5' to 3' end inserted copies without change in the basal levels of expression of this construct. Any further extension of the numbers of CREs resulted in an adversive effect due to squelching of TATA-box mediated basal levels of expression. To circumvent this limitation, we constructed by PCR a construct designated pΔMC16LUC, which contained a duplication of the cAMP-responsive region 5'-CRE_8-TATA-3'.

A panel of cell lines was screened to identify those combining efficient expression from the pRK vector (CMV promoter and CMV enhancer) with high transfection efficiency and with highest responsiveness of the reporter to cAMP. In a preliminary survey, we confirmed in Northern blot experiments that expression from the pRK vector in LLC-PK1 cells was clearly superior to other cell lines used in standard expression cloning techniques e.g. Cos-1 and 293 cells. According to general view highest levels of expression are considered to provide the best chance to detect a specific signal against background noise. Therefore, COS cells are the model of choice in expression cloning strategies allowing replication of transfected cDNAs and resulting in high amounts of proteins of interest, which can be identified by the resp ctive ligand or antibody. Yet, COS cells were poorly responsive to cAMP in regard to induction of the reporter plasmid, so that we investigated in the next step electroporation parameters in LLC-PK1 cells to

obtain high transfection efficiency (number of transfected cells). Electrotransfection parameters (voltage, capacitance, resistance, transfection volume, electrodes, buffer composition) were varied systematically and evaluated semi-quantitatively by *in situ* staining of galactosidase activity of the co-transfected plasmid pCH110, which encodes the β-galactosidase gene under the control of the SV40 promoter. As expected, transient expression levels and transfection efficiency increased linearly to higher field-strengths. In a second series of experiments, we tested the range of induction observed for co-transfection of pΔMC16LUC with a control plasmid encoding a G-protein coupled receptor expressed from the pRK vector. Importantly, the highest induction ratios obtained deviated clearly from the parameters suggested by *in situ* staining. Strikingly, under conditions revealing expression of the marker protein galactosidase in >80% of the cells the response to cAMP was severely impaired in its amplitude. In contrast, those cells revealing moderate levels of unstimulated luciferase activity with typically 40% of the cells being transfected displayed the strongest induction ratios. This finding was further substantiated by the fact that maximal stimulation of the reporter by endogenous vasopressin receptors of the host cell coincides with those settings derived from transfection of a recombinant cDNA encoding a G-protein coupled receptor. Conclusively, highest sensitivity of this system to cAMP is achieved in case recovery following electroporation is maximized, which will by far outpass any advantage of higher levels of transfection efficiency and higher levels of DNA in individual cells. This correlation is acknowledged in the designation functional expression transductory cloning technique (FETCH) to emphasize that identification of target clones depends on expression of functional (full-length) cDNAs, the presence of which is detected by subsequent activation of an endogenous signal transduction pathway and can be monitored by activation of a downstream amplificator, i.e. the reporter gene.

Additional improvements were introduced to reduce further the extent of cell death during electrotransfection and to permit fastest recovery within the time frame pre-set by the decay of the transfected DNA within 48 hr. At this step, cell density proceeding splitting of the cells and in turn numbers s eded proved to

determine decisively cell viability and viability-independent set-points of cAMP-responsiveness. For instance, transfection of a confluent plate of LLC-PK1 cells resulted in slightly increased cell death but an almost complete loss of cAMP-responsiveness of the reporter due to a dramatic upregulation of basal levels of expression equivalent to the activity obtained under the induced state. This result indicates that cell-cell contact and in-turn mitotic activity of LLC-PK1 cells controls responsiveness of cAMP-dependent transcription factors activated by G-protein coupled receptors. Therefore we developed an empirical scheme to passage LLC-PK1 cells: On day one, cells were seeded at a density of $3.3x10^4$ cells/cm^2 and allowed to grow for 48 hr. Since the doubling time is about 18 hr under exponential growth conditions plates are around 75% confluent on day three, on which medium is renewed. This medium change provides a strong growth stimulus and results 24 hr later in a mild growth arrest due to increasing cell density. Cells for electrotransfection were splitted in the evening at $6.6x10^4$ cells/cm^2 and the release from this growth block allowed an enforced mitotic activity 12 hr later with no visible cell death following electroporation, low levels of basal expression of the reporter and an excellent response to stimulation by cAMP. The cells of the stock population were kept under identical conditions (day 1 seeding $3.3x10^4$/cells cm^2 cells, day 3 medium renewal, day 4 passaging), which resulted in an accelerated growth behavior. The transition into an optimized transfection competent state required at least two rounds of passages of LLC-PK1 cells under the detailed protocol.

In addition we tested an array of tools described to enhance DNA-uptake and stability (synchronization of cells, butyrate, PEG) or to enhance the responsiveness of the PKA-pathway (Ca^{++}-ionophores, PKC-agonists, phosphatase inhibitors) with all of them influencing adversively sensitivity due to reduced cell viability. A notable exception of this rule was the omittance of serum 8 hr after electrotransfection. Although serum was required immediately after electrotransfection during the recovery phase, one wash and replenishment with serum-free medium in the evening resulted in a 2-3-fold increase in cAMP-

responsiveness of the system, which was attributable to a lowered basal activity of the reporter.

Following transfection of cDNA pools in LLC-PK1 cells stimulation of endogenous vasopressin receptors and activation of the reporter served as an internal control to evaluate responsiveness of the PKA-pathway and in turn cell viability. Though electroporation reveals a higher reproducibility compared to chemical methods slight variations can considerably distort the interpretation of induction ratios because of the above-mentioned dependence on cAMP-responsiveness. In addition we included as a positive internal control a plasmid encoding the $ß_1$-adrenergic receptor, which is positively coupled to cAMP-production and was expressed from the same expression cloning vector. Identical aliquots of this control plasmid were added to each pool of cDNAs to be tested and a control pool, which was composed of one clearly negative pool of 2,000 independent clones. The combined information of induction ratios for vasopressin and the $ß_1$-agonist isoproterenol allowed to discriminate between the following situations:

a) low ratios for vasopressin and isoproterenol point to low cAMP-responsiveness and impaired cell viability.

b) high ratios for vasopressin and low ratios for isoproterenol point to inefficient transfection or degradation of pool DNA

c) high ratios for vasopressin and isoproterenol point to optimal transfection.

d) isoproterenol ratio of test pool below the one of the control pool indicate a number of clones > 2,000 or a poor quality of the DNA.

e) isoproterenol ratio of test pool above the one of the control pool indicate a number of pools <2,000 leading to an overestimate in the number of independent clones screened.

In the presented scheme the cut-off for each induction ratio obtained for a substance tested has for each pool to be related to the respective ratios obtained for the external vasopressin and the internal isoproterenol control. In this view a PACAP-dependent induction ratio of 3-fold under condition a) has to be considered significant, whereas under condition c) reflects a borderline value.

This standardization allows to compare different samples from the same or different experimental settings and is a prerequisite to compare results from retesting of borderline pools or from successive subdivisions of a putatively positive pool.

Separate aliquots of cells were incubated with peptide hormones, including PACAP, 12 hr after electroporation. One pool of clones from the rat colliculi library consistently stimulated luciferase activity in the presence of PACAP and a functional clone encoding the PVR1 receptor was isolated by successive subdivisions (Spengler et al., Nature 365 (1993), 170-175). Subdivision of the pool of clones was achieved by subdividing the cDNA library until the pool of clones represented a substantially homogeneous pool of clones which consistently stimulated luciferase activity. Several other pools displayed the same phenotype, namely a PACAP-dependent stimulation of the reporter gene and the corresponding active clones were isolated by the same subdivision process. Sequencing was carried out by subcloning restriction fragments in pBSBluescript using T3, T7 and internal primers. Two clones from the AtT-20 library (p2195 and p1270) inducing PVR1 expression turned out to encode the same protein (in the scope of the present invention referred to as Bop1).

The isolated cDNA clones p2195 and p1270 contained a 2.8 kb and 4.7 kb insert, respectively. Entire sequencing of clone p2195 revealed a 2790 bp cDNA (shown in SEQ ID NO. 1) encoding an open reading frame of 667 amino acids (shown in SEQ ID NO. 2) giving rise to a protein with a predicted molecular weight of 75 kDa (Figure 1A). The ATG of AGGCCATGG (SEQ ID NO. 4) was assigned as initiation codon on the basis of its close match to the CC(A/G)CCATGG (SEQ ID NO. 5) Kozak consensus sequence for favored initiation of translation and the presence of an in-frame TGA stop codon 12 nucleotides upstream. Data base searches revealed the presence of seven zinc fingers (Klug and Schwabe, FASEB J. (1995), 597-604) in the N-terminal region of Bop1. However, homologies to other members of the zinc finger protein family were low (30% for the best), with the closest group being the GLI-Krüppel family of zinc finger

proteins which have been implicated in normal development and tumor formation (Ruppert et al., Mol. Cell. Biol. 8 (1988), 3104-3113). In particular, the first H/C link (HSRERPFKC (SEQ ID NO. 6)) is in good agreement with the consensus motif for the GLI-Krüppel family (H(S/T)GEKP(F/Y)XC (SEQ ID NO. 7)) (Schuh et al., Cell 47 (1986), 1025-1032). On the other hand, the remaining 459 C-terminal amino acids displayed no significant homologies to sequences in the Swissprot and NBRF-PIR data bases. The central region of the protein (275-383) is characterized by 34 PLE, PMQ or PML repeats, suggestive of a structure known as poly proline type II helix which is considered to be critically involved in protein-protein interactions (Williamson, Biochem. J. 297 (1994), 249-260). The COOH-terminal region is particularly P-, Q- and E-rich, a feature often displayed by transactivation domains of transcription factors. In addition, the presence of a putative phosphorylation site (H<u>S</u>PQK (SEQ ID NO. 8)) for cyclin-dependent kinases (Cdks) located between the second and third zinc finger motif (residues 56-60) as well as a putative PKA-phosphorylation site (KKW<u>T</u> (SEQ ID NO. 9)) at the very C-terminus (residues 663-666) suggests possible regulation by protein kinases.

Since the cDNAs p2195/p1270 were derived from the AtT-20 tumor cell line there is a potential risk that they harbor mutations which may result in loss or gain of functions not associated with the wild-type form. To rule out this possibility we recloned Bop1 from a plasmid library constructed from whole pituitary tissue of Balb/c mice. To isolate a Bop1 wild-type cDNA, poly(A)$^+$ RNA was obtained from 80 male Balb/c mice (Balb/cAnNCrlBR) and reverse transcription was performed on 5 μg poly (A)$^+$ with a random primer-NotI adapter (5'-ATGTCTCGAGGCCTTTGCGGCCGCTATANNNNNNNN-3' (SEQ ID NO. 3)). After second-strand synthesis, BstXI adaptors (In-Vitrogen) were added. The cDNAs were digested with Not I, size-selected on a chromaspin column 1000 (Clontech) and cloned into the BstXI /NotI sites of pRK8, a modified pRK5 vector (Spengler et al., Nature 365 (1993), 170-175). Screening of ~ 0.5 x10^6 clones with the p2195 cDNA probe allowed the isolation of one full-length cDNA clone designated B-16, which contained a 3.7 kb insert. Transfection of B-16 into LLC-

PK1 cells successfully substituted for p2195 or p1270 with respect to regulation of PVR1 expression. Entire sequencing of clone B-16 showed a 86 bp non translated 5' region and an extended non translated 3' region of 0.7 kb (Figure 1B). The coding region of B-16 was identical to p2195 except the reading frame was interrupted at residue 658 by a 630 bp insertion. The sequences at the boundaries of this insertion are in excellent agreement with consensus exon-intron junction sequences and preserve the reading frame (Figure 1B). We observed this insertion at exactly the same position in clone p1270 derived from the AtT-20 library (Figure 1B). This finding argues against a cloning artefact in clone B-16 and suggests the presence of an unspliced intron region. In support of this hypothesis, a PCR-based fragment encoding the intron region failed to hybridize to a poly-A^+ blot from AtT-20 cells. The distribution of Bop1 was assessed by Northern blot of total RNA prepared from different mouse tissues. Interestingly, the anterior pituitary gland displayed by far the highest level of expression of Bop1 mRNA (Fig. 1C). Bop1 gene was expressed at much lower levels in various brain areas including olfactory bulb, cortex, hippocampus, hypothalamus-thalamus, brain stem and cerebellum, while no hybridization was observed in peripheral tissues.

Example 2: Constitutive Expression of Bop1 and p53 Abates Growth of Tumor Cells

In order to study the function of Bop1 we aimed to generate clones stably expressing Bop1 protein in the LLC-PK1 cell line. However, independently of the resistance marker employed, we failed to establish a Bop1-expressing cell clone. To evaluate the possibility that Bop1 inhibits tumor growth we subcloned Bop1 and p53 in sense and anti-sense orientation downstream of a cytomegalovirus promoter in a vector (pCMVPUR) carrying the puromycin resistance gene.

The pCMVPUR sense/antisense constructs (1.0 μg) and pGEM4 filling DNA (3.0 μg) were transfected into 2×10^6 into the LLC-PK1 cell line and in addition into the

The epithelial cell line LLC-PK1 and the human osteosarcoma cell line Saos-2 were electrotransfected (n=3) with the parent vector pCMVPUR or with vectors encoding sense and antisense Bop1 or wild-type rat p53. pGEM4 carrier DNA replaced pCMVPUR in mock transfected cells. 24 hr later, cells were grown in the presence of 5 µg/ml of puromycin and kept for 10 days with regular medium changes. To score viable colonies cells were incubated with MTT.

Example 3: Bop1 and p53 Suppress Growth of Tumor Cells

A system for tetracycline-regulated gene expression was recently described (Gossen and Bujard, Proc. Natl. Acad. Sci. 89 USA (1992), 5547-5551). This system relies on constitutive expression of a tetracycline-controlled transactivator protein (tTA) which activates target genes placed under the control of a regulatory sequence (tetO). Binding of tetracycline (Tc) or its higher affinity derivative anhydrotetracycline (ATc) to tTA prevents activation, whereas activation is achieved by withdrawal of the repressor (Gossen et al., Trends Biotech. 12 (1994), 58-62). In the approach presented here LLC-PK1 and Saos-2 cell lines are transfected with a tTA-encoding vector and isolated one clone from each cell line (L-tTA and S-tTA) which displayed efficient regulation of genes cloned downstream of the tetO sequence.

In addition, a new cis-regulatory expression vector was developed with distinct lower basal levels of expression and potent regulatory properties equivalent to or exceeding those exhibited by the original minimal CMV-based expression vector in a panel of host lines attesting to a broad use of this system in future applications, most preferably the study of TSGs. The regulatory region of pUHC13-3 (Gossen and Bujard, Proc. Natl. Acad. Sci. USA 89 (1992), 5547-5551) was excised by HindIII and EcoRI partial digest and inserted into pBlueScript SK(-) digested by EcoRI and HindIII to give pBS-CMVtetO. A fragment of 157 bp encoding the tetO sequence was released by SmaI and inserted into the plasmid pΔMTVLUC (Spengler et al., Nature 365 (1993), 170-

human osteosarcoma cell line Saos-2 (ATCC HTB 85), which was previously shown to be growth-inhibited by wild-type p53 (Diller et al., Mol. Cell. Biol. 10 (1990), 5772-5781). pGEM4 replaced pCMVPUR in mock transfected cells. Three electroporations for each construct were pooled and aliquots were plated in 15 cm culture dishes. The cell lines were grown in DMEM (GIBCO) supplemented with 10% fetal calf serum (GIBCO). Selection with puromycin (5.0 µg/ml) was started 24 hr after transfection. Following transfection, cells were grown with puromycin for 10 days, and the number of viable colonies was scored after incubation with MTT. Data presented in Table I show that introduction of Bop1 sense expression vectors resulted in a substantial suppression of colony formation equivalent to that induced by p53. Abrogation of cell growth by Bop1 or p53 was more prominent in the Saos-2 cell line. In addition the clones that did appear after transfection of Bop1 or p53 sense constructs into the LLC-PK1 cell line died when reexposed to selection after passaging and grew at a slow rate in case further selection was omitted.

Table I

Bop1 and p53 Suppress the Growth of Tumor Cells

Cell type	(n)	plasmid	antisense	sense	ratio
LLC-PK1	3	Bop1	1014 ± 170	2	507
	3	p53	1452 ± 258	2	726
	1	vector	1653 ± 270		
	1	mock	0		
Saos-2	3	Bop1	2538 ± 354	1	2500
	3	p53	3779 ± 566	1	3800
	1	vector	4517 ± 641		
	1	mock	0		

175) linearized at +256 bp by HindIII and blunted with T4-DNA polymerase to give the construct pΔMtetOLUC.

The XhoI site (+1) of pΔMtetOLUC was converted into a NotI site by insertion of an oligonucleotide with an internal NotI site. To obtain pΔ5'ΔMtetOLUC a StuI (+863) / NotI (+1) fragment of pΔMtetOLUC was inserted into pBlueScript cut by SmaI/NotI and shortened in size by PpuMI (+786 bp) and EcoRV digestion, blunting and relegation. This fragment was either transferred back into the plasmid pΔMTVLUC using the pBlueScript polylinker HindIII site and the internal BstEII site (+56bp) or transferred into the plasmid pOPIPUR by HindIII and NotI digestion to give PMtetO. The vector pOPIPUR is derived from pOPI3CAT (Stratagene) and contains the puromycin gene of pPUR (Clontech) under the control of the SV40 promoter.

Additional copies of the heptameric tetO sequence were isolated from pBS-CMVtetO by SmaI and KpnI digestion and inserted into PMtetOLUC restricted within the tetO sequence by Ecl136II and KpnI. Using this strategy, a series of constructs with increasing numbers of tetO copies was created, which are abbreviated in the following part as $PMtetO_XLUC$ with the index displaying the number of copies of the heptamer tetO.

Target cDNAs were inserted downstream the ΔMtetO sequences via the unique Not I site. For stable transfections the plasmids p3'SStTA, $PMtetO_5Bop1$ and $PMtetO_5p53$ were linearized with Eam1105II and 1 μg of DNA was co-transfected with 3 μg pGEM4 filling DNA into $2x10^6$ cells. Selection of tTA-cell clones started 24 hr after transfection using hygromycin (MERCK) at a concentration of 700 μg/ml and 500 μg/ml in LLC-PK1 and SaOs-2 cells, respectively. Selection for clones expressing the Bop1 gene or p53 was carried out at a concentration of 5.0 μg/ml puromycin. The following numbers of clones were screened: L-tTA: Bop1=95, p53=92 and S-tTA: Bop1 n=77, p53: n=72. All the clones revealed impaired cell growth to varying degrees under the activated state (-ATc), which was microscopically scored twice during seven days. For each condition one half of the most promising clones was subjected to additional rounds of analysis with about 10 clones remaining at the fourth round.

Three candidate clones from each transfection condition were subjected to a preliminary analysis of counts of cell numbers. The LLC-PK1- and Saos-2-derived clones (L-Bop and L-p53, S-Bop and S-p53, resp.) displaying the greatest differences in growth were further analyzed (Figure 2A). Importantly, no major differences in the growth behavior were observed in the presence of the repressor ATc between Bop1- and p53-expressing clones and the parent clones L-tTA and S-tTA (Figure 2A). Therefore the differences in cell counts on day six were primarily due to the suppression of growth in the absence of the repressor. Measurement of proliferation rate revealed that Bop1 (L-Bop: 11-fold; S-Bop: 20-fold) was slightly less potent than p53 (L-p53: 15-fold; S-p53: 25-fold) in reducing the growth rate of both cell lines. Western blot analysis proved that Bop1 protein was not detectable in L-Bop or S-Bop cells in the presence of ATc. A strong increase in protein levels of Bop1 was noted in the activated state (Figure 5B). Similar results were also obtained for the regulation of p53 in Saos-2 and LLC-PK1 cells (Figure 5B). These results emphasize that the modified expression vector combines low basal activity with potent regulatory properties.

Total counts of cell numbers do not necessarily discriminate between alteration of cell proliferation and viability. It was therefore decided to evaluate the effects of Bop1 and p53 expression by two complementary methods. First, DNA-synthesis was studied with a non-radioactive immunoassay based on incorporation of 2-bromodeoxyuridine (BrdU) into nuclear DNA on each of six days with or without ATc (Figure 2B). Second, it was measured the conversion of the tetrazolium salt MTT to formazan blue, which depends on the activity of mitochondrial and cytoplasmatic dehydrogenases. This activity depends on cell viability and closely correlates with cell proliferation (Figure 2B).

The counts of Cell Numbers, 2-Bromodeoxyuridin incorporation and Formazan production were performed as follows:

Equal number of cells (5,000) were seeded in 24-well plates in DMEM / 10%FCS supplemented with ATc (10^{-11} µg/ml). After recovery for 36 hr, medium was renewed and the repr ssor omitted for half of the samples. For samples lacking the repressor, the medium was changed again 3 hr later to remove residual

amounts of ATc. Growth medium was changed routinely on day 3. Average cell counts from 3 to 5 experiments in triplicate are plotted versus time after removal of the repressor. For measurement of DNA-synthesis cells (1,000) were seeded in 48-well plates and cultured as outlined above. On each of six days, 10 μM 2-bromodeoxyuridin was added for 8 hr and subsequent steps were carried out according to the manufacturer's instructions (Boehringer Mannheim). For measurement of cell viability, 1,000 cells were seeded in 24-well plates and cultured as described above. The average of OD measurements for DNA-synthesis and cell-viability was obtained from three experiments performed in triplicates. To test serum-independence, cells were kept in normal medium for 36 hr before serum was washed out once with DMEM and replaced by DMEM / 0.1%FCS / ±ATc.

The results obtained for S-Bob and S-p53 emphasize the observed differences in cell counts (Figure 2A), which correlate with those obtained in overall cell proliferation and overall viability measurements (Figure 2B). Similar results were obtained for L-Bop and L-p53. Cells from LLC-PK1 and Saos-2 clones kept under low serum conditions (0.1%FCS) in the repressed state displayed reduced growth rate and cell death from day three on, indicating serum-dependence to maintain logarithmic growth (Figure 2C). In contrast, proliferation under expression of Bop1 and p53 remained unchanged (Figure 2C). Therefore, inhibition of tumor growth by Bop1 and p53 proceeds through mechanisms unrelated to the presence of serum factors in these cellular models.

The ability of Bop1 to suppress growth could be due to a non-specific lethal effect of protein overproduction, resulting in cell death. Alternatively, it could be a manifestation of a more specific effect on cell proliferation. To further investigate these two possibilities, the growth pattern following reexposure to ATc of the surviving cells was tested. The impairment of cell growth by Bop1 and p53 expression was transient for both the LLC-PK1 and Saos-2 clones studied. Reexposure to the r pr ssor ATc caused cells to resume logarithmic growth after 48 hr (Figure 2D). Therefore, Bop1- and p53-induced changes in cell growth were

not permanent and at least in part reversible, arguing against a non-specific effect of protein overproduction.

Example 4: Bop1 and p53 Inhibit Soft-Agar Colony Formation

Anchorage-independent growth is often correlated with tumorigenesis and is a strong criteria for cultured cell transformation. To test the influence of Bop1 or p53 on anchorage-independent growth, LLC-PK1 and Saos-2 cell clones were assayed for their ability to grow in soft-agar. Each well (35-mm) of a six-well culture dish was coated with 4 ml of bottom agar mixture (DMEM/10%FCS/0.6% agar/±ATc). After the bottom layer had solidified, 2 ml of top agar mixture (DMEM/10%FCS/0.3%agar/±ATc) containing the cells was added. ATc was used at a final concentration of $3x10^{-11}$ µg/ml. After 7 days, another 1.5 ml top agar mixture (±ATc) was added. On day 10, the wells were overlaid with 2 ml MTT (1mg/ml) and incubated for an additional 4 hr, washed once with PBS and then photographed. Colony formation by Bop1 or p53 expressing cells (-) was dramatically reduced compared to the repressed state (+) (Figure 3). Also the few colonies formed under Bop1 or p53 expression were of smaller size. These results demonstrate that Bop1 and p53 can abate anchorage-independent growth of tumor cells, one of the hallmarks of tumorigenicity.

Example 5: Bop1 and p53 Suppress Tumor Formation in Nude Mice

The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Yet, not all of the altered cellular growth properties commonly associated with the transformed state in-vitro are required for neoplastic growth in-vivo and vice versa. Therefore loss of tumorigenicity under expression of Bop1 in-vivo would be a critical test to substantiate the tumor suppressor function of Bop1. To achieve gene regulation by Tc in nude mice, half of the animals were implanted with Tc pellets whereas the remainder were implanted with placebo pellets. 36 nude mice were randomly

distributed into three groups of 12 animals. In each group, half of the animals were subcutaneously implanted with Tc pellets (63 mg; 0.7 mg tetracycline hydrochloride per day; Innovative Research of America) and the remaining half were implanted with the placebo pellets (Innovative Research of America). Two days latter, each animal was injected subcutaneously on each side with S-Bop or S-p53 cells which were grown in the presence of ATc, trypsinized and resuspended in PBS at a density of $5x10^7$ cells/ml. 100 µl of this cell suspension was injected subcutaneously into each side of each animal grown in the continuous presence of ATc. Two groups were injected with S-Bop cells from two independent trypsinizations whereas one experiment was performed with S-p53 cells. Due to the clonal origin of S-Bop and S-p53, differences in the tumorigenicity of each clone were observed as inferred from the difference in the observed lag in tumor formation which was assessed at 11 weeks after cell injection for S-Bop and at 16 weeks for S-p53. S-Bop- and S-p53-injected animals were sacrificed at 11 and 16 weeks, respectively, dissected and the tumors were weighed. Table II presents results from two experiments with S-Bop (Bop1) and one experiment with S-p53 (p53). In agreement with previous results (Chen et al., Science 250 (1990) 1576-1580), p53 expression impaired tumor formation by Saos-2 cells in-vivo. Interestingly, Bop1 was as efficient as p53 in inhibiting tumor formation as deduced from tumor incidence (Table II) and from the average tumor weight (193±13 mg (n=14) for Tc vs. 18±7 mg (n=2) for placebo). Conclusively, Bop1 and p53 are equipotent at inhibiting tumor formation in-vivo.

Table II

Bop1 and p53 Inhibit Tumor Formation in-vivo

clone	tumor incidence (No. of tumor-bearing injection sites / total No. of injection sites)	
	placebo	Tc
S-Bop (Bop1) exp. n°1	2/12	14/14
S-Bop (Bop1) exp. n°2	1/12	12/12
S-p53 (p53)	1/12	10/12

Nude mice were implanted with placebo or Tc pellets subcutaneously. Two days latter, $5x10^6$ cells from each clone were injected subcutaneously into each side of each animal, and tumor formation was scored at 11 weeks for S-Bop (Bop1) and 16 weeks (p53).

Example 6: Expression of Bop1 and p53 induce apoptosis

Two days following induction of p53 expression, Saos-2 cells flattened and greatly enlarged (three to eight fold) in average diameter, which was most evident when grown in small clusters. Similar changes, though less prominent (two to fourfold increases in the average diameter), were also observed for L-p53. In contrast, Bop1 expressing LLC-PK1 or Saos-2 clones appeared indistinguishable from the parent cell lines giving a first hint of functional differences between Bop1 and p53. Yet, an increasing number of cells with signs of lost cell viability was observed from day two onwards following Bop1 or p53 expression. These cells failed to convert MTT, shrank in size, were abundant in phase contrast microscopy, revealed membrane blebbing, and rounded further up before detaching from the plates. For Bop1 these alterations were most evident in Saos-2 cells (S-Bop) and

for p53 in LLC-PK1 cells (L-p53) and appear reminiscent of an apoptotic cell death. This form of cell death is often accompanied by fragmentation of the DNA into a ladder of regular subunits.

To address this question LLC-PK1 and Saos-2 cells were seeded with (4,000 cells/cm^2) or without (8000 cells/cm^2) ATc for 3 days and soluble DNA was prepared as described (Hockenbery et al., Nature 348 (1990), 334-336). Aliquots of DNA were fractionated on a 1.2 % agarose gel. When the repressor was omitted a clearly visible degradation into oligonucleosomal DNA fragments became evident (Figure 4 A), which was most advanced following expression of Bop1 in Saos-2 cells.

The fluorescent DNA-stains ethidium bromide and acridine orange were employed to examine nuclear changes under the ATc-deprived condition. Therefore, the cells (5x10^4) were seeded in the absence of ATc in 12-well clusters and grown for three days. After aspirating the medium, the cells were washed twice with PBS and overlaid with a staining mix of ethidium bromide (50 µg/ml) and acridine orange (10 mg/ml) for 10 - 20 min. Photography was carried out using UV-filters of 400-420 nm and of 510-550 nm.

Since the flattened and enlarged cell shape of p53-expressing cells enhanced attachment to the plastic surface, a comparable large population of cells exhibited nuclear signs of apoptosis, whereas Bop1-expressing cells shrank, dislodged quickly and appeared less represented in these experiments. The structural changes of nuclear demise following Bop1 expression were even more evident when floating cells were collected and subjected to analysis (Figure 4B). Decay of the nucleus involved nuclear shrinkage, condensation of the chromatin, collapse into patches and then into crescents in tight apposition to the nuclear envelope, and finally in one or several dense spheres (Figure 4B).

To investigate the extent of DNA-damage, terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed using the ApopTag kit (Oncor), according to the manufacturer's instructions, followed by flow cytometry. The cells were seeded with (1,800 cells/cm^2) or without (3,600 cells/cm^2) ATc for 3 days. Then the cells were pelleted, kept on ice for at least 10 min and

resuspended in 900 µl of propidium iodide staining solution (PISS = 50 µg/ml propidium iodide; 0.1 % trisodium citrate dihydrate; 0.1 mg/ml RNase A; 0.1 % Triton X-100). Following an overnight incubation, cell cycle phase distribution was determined with FACScan (Becton-Dickinson) with 20,000 events analyzed using Modfit software (Verity Software House, Inc.). Incubation of each clone in the absence of ATc induced a large increase in the number of cells with enhanced or high fluorescence indicative of free DNA ends and nuclear-fragmentation (Fig. 4C). These results confirm the data obtained with ethidium bromide-stained gels and indicate that the proportion of cells displaying nuclear damage was as high as 60-70 % following expression of either Bop1 or p53.

Taken together these experiments give convincing evidence that Bop1 and p53 recruit apoptotic programs to inhibit growth of tumor cells and Saos-2 cells seem highly apoptosis proficient following expression of Bop1.

Example 7: Expression of Bop1 and p53 induces changes in cell cycle distribution

To characterize further the mechanisms by which Bop1 might regulate cell growth the distribution of cell cycle phases was studied. Increases in wt p53 levels are known to suppress cell growth by blocking the cell cycle at the G1 to S transition (Hunter and Pines, Cell 79 (1994), 573-582; Sherr and Roberts, Genes and Dev. 91 (1995), 1149-1163). More recently p53 has been suggested to address an additional checkpoint by arresting cells at the G2/M boundary (Agarwal et al., Proc. Natl. Acad. Sci. USA 92 (1995), 8493-8497; Cross et al., Science 267 (1995), 1353-1356; Stewart et al., Oncogene 10 (1995), 109-115; Yamato et al., Oncogene 11 (1995), 1-6). In control experiments, the parent clones L-tTA and S-tTA showed no difference in the distribution of cells in different phases of the cycle in the absence or presence of ATc. In contrast, expression of Bop1 reduced the proportion of S-Bop populations in S phase and G2/M phase from 37.8 % and 17.5% to 24.5 % and 12.6 %, respectively. Importantly there was a clear

compensatory increase of cell populations in G1 from 44.7 % for the repressed state to 63.0 % for the expressed state of S-Bop (Figure 5A).

The results obtained for p53 expression in the S-p53 cell clone are in agreement with those obtained recently with a temperature-sensitive mutant p53 in Saos-2 cells (Yamato et al., Oncogene 11 (1995), 1-6). A decrease in G1 and S phase from 39.4 % to 31.8 % and from 43.7% to 35.0 % was observed and a clear increase in G2/M from 16.9 % to 33.2 %. (Figure 5A). The failure of p53 to proceed to a G1 arrest reflects most likely the presence of the deleted non-functional retinoblastoma gene product (Rb) in the Saos-2 cell line.

These observations were extended to the LLC-PK1 cell line and though shifts of populations in cell cycle phases under expression of Bop1 and p53 were less prominent than in the Saos-2 cell clones, there was again a clear increase in G1 phase populations for expression of Bop1 (G1 59.1 % vs. 43.7 %; S 28.2 % vs. 38.9 %;G2/M 12.7 % vs. 17.4 %) and a shift for G2/M populations under p53 (G1: 39.3 % vs. 44.1 %; S: 32.1 % vs. 40.2 %;G2/M: 28.6 % vs. 15.7 %) .

p53 achieves G1 arrest through transactivation of the gene encoding the cyclin-dependent kinase inhibitor p21 (also designated Cip1, Waf1, Sdi1, Cap20). Increased levels of p21 inhibit the kinase activity of cdk2 and maintain Rb in its underphosphorylated state in tight association with members of the E2F family. As a result, transactivation of genes driving the cell cycle is inhibited (Goodrich et al., Cell 67 (1991), 293-302; Weinberg, Cell 81 (1995), 323-330). The question arose whether Bop1-induced G1 arrest utilizes the same molecular pathway as p53. Expression of p53 in Saos-2 cells resulted in a strong induction of the p21 protein proving an intact and efficient transactivation of the endogenous gene by the exogenous p53 protein (Figure 5B). Yet, no regulation of the p21 gene in Saos-2 cells was encountered following expression of Bop1 (Figure 5B). The same results were obtained in the LLC-PK1-clones with a strong induction of p21 by p53. Conclusively, Bop1 induces G1 arrest in these cellular models through molecular relays independent of p21.

In a number of cellular systems, wt p53 activation has been shown to confer growth arrest (Mercer et. al., Proc. Natl. Acad. Sci. USA 87 (1990), 6166-6170;

Merlo et al., Oncogene 9 (1994), 443-453; Michalovitz et al., Cell 62 (1990), 671-680; Roemer and Friedmann, Proc. Natl. Acad. Sci. USA 90 (1993), 9252-9256). In contrast, wt p53 failed to cause a measurable arrest in M1 cells and cell cycle progression proceeded while viability was lost within 48 hr (Yonish-Rouach et al., Mol. Cell. Biol. 13 (1993) 1415-1423; Yonish-Rouach et al., Nature 352 (1993) 345-347). In that system, cells in G1 appeared to be preferentially susceptible to the death-inducing activity of wt p53. Therefore the question arose whether in the used cellular models, in which Bop1 and p53 play a dual role in regulation of apoptotic cell death and cell cycle progression, a particular phase of the cycle is associated with protection or increased susceptibility to cell death. To address this issue the cell cycle analysis was extended and double staining with propidium iodide was performed to measure DNA content and TUNEL to assess apoptosis. As shown in Fig. 5C, apoptotic cells proceeded from each phase of the cell cycle as indicated by the distribution of DNA content of apoptotic cells. It was concluded that cell cycle arrest is not a prerequisite to apoptosis and that both Bop1 and p53 induced apoptosis through a pathway which is independent of the one involved in cell cycle arrest.

Example 8: Bop1 is a nuclear transcription factor

Structural analysis of Bop1 demonstrated features compatible with a transcription factor composed of a N-terminal seven zinc finger DNA-binding domain and a COOH-terminal transactivation domain. Without information on the actual cis-regulatory sequences recognized by Bop1 to transactivate target genes, it was decided to use the induction of the endogenous PVR1 gene as a model to dissect functional domains of Bop1. A bimodal regulation of the PVR1 gene was observed, indistinguishable for Bop1 and wt p53 cDNAs as measured by induction of the cAMP-sensitive luciferase gene (Figure 6B). The decrease in PVR1 expression with high amounts of cDNAs was unrelated to cellular toxicity. The two-zinc finger domain of the hybrid steroid receptor GM_2M (Rupprecht et al.,

Mol. Endocrinology 7 (1993), 597-603) was replaced with the seven-zinc finger domain of Bop1 (B_Z) to create GB_ZM (Figure 6A).

The hormone-binding domain of the mineralocorticoid receptor was replaced in this construct to avoid possible pleiotropic effects associated with glucocorticoids.

The GR_{NX}, MR_{NX} and GM_ZM constructs were previously described (Rupprecht et al., Mol. Endocrinology 7 (1993), 597-603). Primers used to create GB_ZM were:

5'-gtgatggcggccgCCATTCCGCTGTCAAAATGTG-3' (+7 bp to +27 bp) (SEQ ID NO. 10)

and 5'- ccgcgcctcgagGGTCTTCTTGGTGTGACG-3' (+618 bp to +601 bp) (SEQ ID NO. 11).

The different constructs were subcloned into pRK5PUR. To create the construct ΔB_ZM, the GR-transactivation domain and part of the Bop1 zinc finger binding domain was excised from GB_ZM by EcoRI/MluI digestion and replaced by the restriction fragment EcoRI/MluI (-541 bp to +272 bp) of p2195.

Primers used to create Bop_ZM were:

5'-gcggccgCAGAGCCGTCTTTCACTC-3' (+1148 bp to +1166 bp) (SEQ ID NO. 12) and

5'-ccgcgcctcgagAACTGTCCATTTCTTATAGAC-3' (+2001 bp to +1980 bp) (SEQ ID NO. 13).

The stop codon of p2195 was replaced by the amino acid histidine (CTC) as part of the XhoI site used to ligate to the MR-hormone binding domain. PCR-generated fragments were sequenced to verify accurate amplification.

In transfection of LLC-PK1 cells ($2x10^6$), pGEM4 plasmid was used as carrier and the amount of pRK expression vector was kept constant with pRK5CAT. Luciferase activity was determined as previously described (Spengler et al., Nature 365 (1993), 170-175) 12 hours after transfection.

The Bop1/steroid-receptor hybrid gene GB_ZM was co-transfected with the cAMP-responsive reporter pΔMC16LUC into LLC-PK1 cells. Aliquots of transfected cells w re incubated either with the mineralocorticoid receptor antagonist spironolactone or the agonist aldosterone and PACAP was added to both

conditions after 12 hr (Figure 6B). Though the transactivation potency of GB_ZM was 10-fold less compared to the native Bop1 cDNA, a bimodal induction of the PVR1 gene for increasing amounts of GB_ZM was consistently observed, which closely paralleled the one observed for Bop1 and p53. In contrast the construct ΔB_ZM, which lacks the glucocorticoid receptor transactivation domain failed to confer regulation of PVR1, implicating an active transcriptional mechanism underlying this response (Figure 6C). No regulation of the PVR1 gene was observed for the transfected parent construct GM_ZM.

Further support for the role of Bop1 as a nuclear transcription factor was obtained with the fusion protein Bop_XM, in which the C-terminus of Bop1 was linked with the hormone-binding domain of the mineralocorticoid receptor (Figure 6A). Transfection of this construct into LLC-PK1 cells completely prevented transactivation of the PVR1 gene in the absence of mineralocorticoid receptor ligands. In contrast aldosterone and spironolactone allowed efficient regulation of the PVR1 gene (Figure 6C). The activation of Bop_XM by the aldosterone antagonist spironolactone supports the view that the attached hormone-binding domain merely serves to trap this fusion protein to cytoplasmatic heat shock proteins (Picard, Trends Cell Biol. 3 (1993), 278-280) and does not interfere otherwise with the functions of Bop1. In contrast, release of Bop1 from this cytoplasmatic anchor by either aldosterone or spironolactone allowed nuclear translocation and transactivation of Bop1 targeted genes.

Moreover, to prove nuclear localization of Bop1 immunocytochemistry on S-Bop cells was performed with an antiserum that was raised against a Bop1 fusion protein truncated for the zinc finger domain (GST-BopΔZF).

The plasmid encoding the GST-BopΔZF fusion protein was constructed by a partially digesting the plasmid pRK8-p2195 with BstX I, blunt-ending with T4 DNA polymerase and digesting with Not I. The resulting 0.9kb fragment was subcloned into pGEX-5X-3 (Pharmacia) previously digested with Sma I and Not I. The fusion protein was purified by affinity chromatography using glutathione-sepharose beads followed by SDS-PAGE and electroelution. Rabbits were immunized with

40 µg of the fusion protein and antisera were collected on a weekly basis. Purified IgG were used for western blots and immunocytochemistry experiments. Western blots were performed on total cell lysates (50 µg) with the above-mentioned purified IgG or with commercially available antibodies to p53 (Pharmingen, San Diego, USA catalog # 14091A), p21*Waf1* (Transduction laboratories, Lexington, USA, catalog # C24420), p27*Kip1* (Transduction laboratories, catalog # K25020) and p16*ink4* (Santa Cruz Biotechnology, Inc., Santa Cruz, USA, catalog # sc-759). Immunocytochemistry, and labeling and staining of actin filaments with rhodamine-conjugated phalloidin were performed as previously described (Ibarrondo et al., Proc. Natl. Acad. Sci. USA 92 (1995), 8413-8417). As shown in Figure 6D, no Bop1 immunoreactivity was detected in the presence of ATc whereas an intense nuclear immunostaining was seen in the absence of Atc.

A nucleic acid molecule prepared by the process described herein is exemplified by a culture deposited in the culture collection Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig, Germany on August 12, 1996, and identified as:

pBluescript II SK (-) p2195 (NotI).

This culture was assigned accession number DSM 11112.

Example 9: Cloning of the human homolog of Bop1

As has been shown in the examples above, Bop1 (in the following referred to as mZac for mouse zinc finger protein which shared with p53 the ability to regulate concomitantly apoptosis and cell cycle progression) is the first gene beside p53 which regulates the two fundamental genetic programs, i.e. cell cycle progression and apoptosis; see Examples 6 and 7. The inventors therefore expected that mZAC should also share with p53 its tumor suppressor activity and isolated the human homolog of mZAC in order to investigate whether human tumors could harbor inactivating mutations of ZAC. Furthermore, hZAC could display otherwise

functional inactivation by methylation of both alleles or imprinting of one allele and loss of the remaining alleles as has been shown for the cdk inhibitor p16 and the new TSG p73 (Merlo et al., Nature Medicine 1(7) (1995), 686-692; Kaglrad et al., Cell 90(4) (1997), 809-819). Therefore, studies on ZAC expression in human tumors are under progress. In addition, functional inactivation of the ZAC gene in human tumors could rely on genetic mechanisms described so far observed only in genetic diseases, such as minisatellite instability in coding and noncoding parts of the ZAC gene (Buard and Jeffrey, Nature Genetics 15 (1997), 327-328). The human counterpart of mZAC (hZAC) was isolated from human brain and pituitary cDNA libraries. $1x10^6$ clones from a human pituitary gland cDNA library (5'-STRETCH, Clontech) were screened with a random-primed probe corresponding to the coding sequence of SEQ ID NO: 1 using standard procedures. 40 positive clones were subcloned into pBlueScript and sequenced on both strands using T3, T7 and internal primers. One 2.3 kb clone comprising the nucleotide sequence given in SEQ ID NO: 16 contained the full coding sequence of hZAC (1389 bp, encoding the amino acid sequence as depicted in SEQ ID NO: 17), with a 802-bp 5' untranslated region and a 142-bp 3' untranslated region. Sequence alignments and phylogenetic trees were performed using Lasergene software, DNASTAR Inc., Madison, WI.

The overall identity between hZAC and mZAC coding sequences was 74.6% at the nucleotide level and 68.5% at the amino acid level. Several domains could be identified in hZAC, each having its counterpart in mZAC (Fig. 7A, 7B). The 7 (C2H2) type zinc fingers (ZF) domain at the N terminus was the most conserved (84.2% similarity at the amino acid level); it displayed the same consensus phosphorylation site for Cyclin-dependent kinases (HSPQK, SEQ ID NO: 8) between the second and third ZF (residues 56-60). The linker region, the Pro, Gln and Glu rich region and the C terminus (Fig. 7B) were rather conserved (54%, 58% and 63% identity, respectively). The last 11 C terminal residues were identical (Fig. 7A). There were two major differences between the mouse and human sequences. Indeed, two regions of mZAC were missing in hZAC: a 34 Pro-repeats (PLE, PMQ or PML) domain and a Glu-clusters domain (Fig. 7A, 7B).

A FASTA analysis for homology search in the Uni-Gene database (05/29/97, (Pearson, Proc. Natl. Acad. Sci. USA 85 (1988), 2444-2448) indicated that hZAC sequence matched 43 human expressed sequence tags derived from various adult sources including placenta, aorta, ovary, prostate, heart, as well as fetal tissues including heart, brain, cochlea, liver and spleen. During the course of this study, a rat and a human sequences designated LOT1 (accession no. U72620 and U72621) were reported (Abdollahi, Oncogene 14 (1997), 1973-1979, Abdollahi, Cancer Res. 57 (1997), 2029-2034) and displayed significant homology to hZAC. Indeed, hZAC and hLOT1 were identical except at 2 residues (Leu^{81} in hZAC and rLOT1 is a Phe in hLOT1 and Pro^{440} in hZAC (Pro^{671} in rLOT1) is an Ala in hLOT1). Suprisingly, the 5'-untranslated regions of hLOT1 and hZAC were completely different 189 nucleotides upsteam the ATG. One explanation for this discrepancy is that hZAC or hLOT1 5' ends could correspond to two different splice variants or either one could contain an unspliced intron. The second hypothesis was favored as a putative 3' splicing site (CACAG) was present 190 nucleotides 5' of the ATG in hLOT1. Furthermore, an intron was also present at that position in the mouse gene.

Since large domains of mZAC were missing in hZAC, the existence of additional genes closely related to mZAC was evaluated, which could contain these domains. Extensive PCR analysis of human genomic DNA was performed with degenerate primers, corresponding to residues conserved between hZAC and mZAC in the ZF domain and the Pro and Gln rich region. Only two classes of PCR fragments different from ZAC were isolated but suggestive of closely related genes. During the course of this study, sequences of the corresponding cDNAs became available in the Unigene database. One class of PCR fragments corresponded to hPLAG1, an embryonic gene which could be involved in the pathogenesis of pleiomorphic adenomas of the salivary glands (Kas, Nature Genet. 15 (1997), 170-174). The second class of PCR fragments corresponded to KIAA0198, a cDNA cloned from human cell line KG-1, whose function is still unknown (Nagase, DNA Res. 3 (1996), 17-24). Multiple sequence alignment

revealed that these cDNAs are members of the same new family of ZF proteins (Fig. 7C).

Example 10: Chromosomal localizations and Southern blots of genomic DNAs

First chromosomal localization of both genes were performed by fluorescent in situ hybridization (FISH). The 2.3 kb cDNA of hZAC, the 4.7 kb cDNA of mZAC and the 6 kb BglII fragment of mZAC gene were used as probes, labeled by nick translation with biotin-11-dUTP and hybridized to human and mouse chromosomes as previously described (Eychène, Oncogene 7 (1992), 1657-1660). Detection of hybridization was performed using goat anti-biotin antibodies (Vector laboratories, Burlingame, CA) and rabbit FITC conjugated anti-goat antibodies (Biosys, Compiègne, France). Direct banding of BrdU substituted chromosomes (Lemieux, Cytogenet. Cell Genet. 59 (1992), 311-312) was stained with propidium iodide for human chromosomes and DAPI for mouse chromosomes. Metaphases were observed under a fluorescent microscope (DMRB, Leica, Germany). Images were captured using a cooled photometrics CCD camera and Quips-smart capture software (Vysis).

The hZAC cDNA probe revealed recurrent single and double spots on human chromosome 6 (band 6q25); out of 30 metaphases, 70% exhibited at least one spot in this position (Fig. 8A). cDNA and genomic mZAC probes exhibited recurrent spots on mouse chromosome 10 (band $10A_2$); out of 25 metaphases, 50% showed recurrent simple and double spots in this position with the cDNA probe and 60% with the genomic probe, with low background (Fig. 8A). The mouse genomic probe revealed recurrent single spots on human chromosome 6 band 6q25 with the rate of 20% with low background (Fig. 8A). Since mouse $10A_2$ and human 6q25 loci are syntenic (Copeland, Science 262 (1993), 57-66) and since the mouse probe displayed the same spots as the human probe on human 6q25, mZAC and hZAC were either orthologs or related genes clustered in syntenic regions. This data was confirmed by the FISH mapping of hLOT1 which

was recently published (Abdollahi, Oncogene 14 (1997), 1973-1979). According to the human genome map (Dib, Nature 380 (1996), 152-154, Schuler, Science 274 (1996), 540-546), hLOT1 (hZAC) was located between markers D6S308 and D6S978 at 6q24. Chromosome 6 is the fourth most frequently rearranged chromosome in human tumors (Teyssier, Anticancer Res. 12 (1992), 997-1004). Allelic loss at 6q24 has been reported in B-cell non-hodgkin's lymphomas (Zhang, Genes Chromosomes Cancer 18 (1997), 310-313, Johansson, Blood 86 (1995), 3905-3914) and many solid tumors such as gastric carcinomas (Queimado, Genes Chromosomes Cancer 14 (1995), 28-34), pancreatic adenocarcinomas (Griffin, Cancer Res. 55 (1995), 2394-2399), renal cell carcinomas (Thrash-Bingham, Proc. Natl. Acad. Sci. USA 92 (1995), 2854-2858), astrocytomas (Liang, Neurology 44 (1994), 533-536), melanomas (Millikin, Cancer Res. 51 (1991), 5449-5453, Walker, Int. J. Cancer 58 (1994), 203-206), ovarian carciomas (Lastowska, Cancer Genet. Cytogenet. 77 (1994), 99-105, Foulkes, Br. J. Cancer 67 (1993), 551-559) and breast cancers (Fujii, Genes, Chromosomes & Cancer 16 (1996), 35-39, Noviello, Clinical Cancer Res. 2 (1996), 1601-1606, Theile, Oncogene 13 (1996), 677-685). Moreover, a frequently deleted region at 6q24 defined by D6S292-D6S310-D6S311 around hZAC locus has been identified in breast carcinomas (Noviello, Clinical Cancer Res. 2 (1996), 1601-1606). In addition, microcell-mediated transfer of a defined chromosome 6q fragment around D6S310, thus including hZAC locus, has been shown to suppress the tumorigenicity of the breast cancer cell line CAL51 (Theile, Oncogene 13 (1996), 677-685). These genetic data, suggesting that at least one TSG is localized close to hZAC locus, together with the functional data of the present invention point to hZAC as a candidate TSG involved in breast cancer and located at 6q24.

The data obtained in accordance with the present invention have been strengthened by a study recently published by Abdollahi (Abdollahi, Cancer Res. 57 (1997), 2029-2034) who cloned ZAC through its loss of expression in a rat model of epithelial ovary cancer and thus named it "LOT" for "Lost On Transformation" (Abdollahi, Cancer Res. 57 (1997), 2029-2034). They further isolated hLOT1, which was identical to hZAC, and showed that hLOT1 (hZAC)

expression was also lost in some human ovary cancer cell lines (Abdollahi, Oncogene 14 (1997), 1973-1979). This again suggests that hLOT1 (hZAC) is a candidate TSG.

To further confirm mZAC and hZAC were orthologs, we carried out Southern blots of digested human and mouse genomic DNAs. Human genomic DNA from peripheral blood lymphocytes (obtained from the Montpellier blood bank, France) and mouse genomic DNA from liver were prepared according to standard protocols. Southern blots of digested genomic DNAs were first hybridized with a mZAC probe corresponding to mZAC ZF and linker regions. After autoradiography, blots were stripped and reprobed with a hZAC probe corresponding to hZAC ZF and linker regions. Probes derived from the ZF domains of mZAC and hZAC hybridized to exactly the same bands in both human and mouse DNA (Fig. 8B), indicating that both mZAC and hZAC probes recognized only one gene in both species.

Example 11: hZAC expression in human tissues

hZAC mRNA distribution was determined using a Human RNA Master Blot (PT3004-1, Clontech, Palo Alto, CA) containing normalized amounts of polyA$^+$ RNAs from human adult and fetal tissues hybridized with a hZAC probe according to the manufacturer instructions. Since polyA$^+$ RNA samples (80 to 400ng) on Master blot have been normalized to the mRNA expression levels of eight different house keeping genes, the relative expression levels of hZAC mRNA could be assessed. hZAC was widely expressed in both adult and fetal tissues (Fig. 9). The strongest levels of expression were observed in the pituitary gland, kidney, placenta, adrenal gland. Uterus, mammary gland, ovary, lung, gastrointestinal tract and lymphoid tissues also revealed strong hybridization signals. Skeletal muscle, peripheral leucocytes, liver, whole brain and spinal cord weakly expressed hZAC. In adult brain, the strongest signals were observed in the occipal lobe, the cerebral cortex and the thalamus (see legend of Fig. 9).

Exampl 12: hZAC is a nuclear transactivator

The human osteosarcoma cell line SaOs-2 was grown and electroporated as described in Example 2. Except for the GAL4 fusion proteins, all cDNAs were subcloned into the pRK5 vector (Spengler, Nature 365 (1993), 170-175), 5' untranslated regions were excised and a HA epitope tag was added at the N terminus. Every construct was verified by sequencing.

The intracellular localization of hZAC was determined by immunocytochemistry. For immunocytochemistry, transfected cells were grown on glass cover slips, fixed, permeabilized, incubated with the anti-HA antibody and then with an anti-mouse FITC (Sigma, StLouis, MO). The nuclei of SaOs-2 cells expressing mZAC or hZAC were strongly labeled (Fig. 10A). No signal was detected neither in mock-transfected cells nor in non-permeabilized cells.

To ask whether hZAC was capable of transcriptional activity like many ZF proteins and p53, the transcriptional activation of the luciferase reporter, driven by a minimal promoter sensitive to the yeast transcription activator GAL4 was measured, after transfection of plasmids encoding hZAC or mZAC fused to the GAL4 DNA binding domain. For the GAL4 fusion protein, mZAC and hZAC coding sequences were inserted into the BamHI site of pSG424 (Blau, Mol. Cell. Biol. 16 (1996), 2044-2052). Different amounts of these expression vectors were transfected together with the reporter plasmid pEIBTATALUC (0.5µg), which contains five copies of the GAL4 DNA-binding site upstream of the the EIB-adenovirus derived TATAA box driving expression of the luciferase gene. The co-transfected plasmid pCH110 encoding the ß-galactosidase gene driven by the SV40 promoter served to standardize luciferase values on transfection efficiency.

Transfection of increasing amounts of the fusions containing either mZAC or hZAC induced a stepwise increase of the luciferase activity (Fig. 10B). The GAL4 DNA-binding domain (GAL1-147) alone was devoid of any transactivation activity (Fig. 10B). Fusions of the GAL4 DNA binding domain with the well characterized

transactivation domains of the transcription factors SP1 and CTF (Blau, Mol. Cell. Biol. 16 (1996), 2044-2052) served as positive controls (Fig. 10B).

Example 13: Expression of hZAC inhibited the growth of tumor cells

24h after transfection of hZAC, a reduced number of cells was observed, compared to mock transfected cells. Furthermore, after hZAC transfection, a substantial number of cells displayed signs of lost viability such as cellular shrinkage, blebbing, and condensed chromatin, as it was previously noted for p53 (Bates, Curr. Opin. Genet. Dev. 6 (1996), 12-19) and mZAC, see Example 6. To evaluate whether hZAC had the same antiproliferative properties as its mouse counterpart a colony formation assay was carried out. For colony formation assay, we cotranfected pRK5-PUR containing the puromycine-resistance gene under the control of a CMV promoter (0.2µg) with either mZAC (1 µg), hZAC (3.8 µg), or p53 (1µg) in sense and anti-sense orientations into 2 millions of SaOs-2 cells. The cells from three transfections were pooled and splitted in different plates. After 9 days of puromycin treatment, the clones were MTT stained and counted.
When either hZAC, mZAC or p53 was expressed, the puromycine-resistant clones were smaller than in the control experiments, mZAC or p53 strongly decreased the number of puromycine-resistant colonies (Fig. 11), whereas the antisense constructs had no effect. hZAC also inhibited cell growth although less strongly (Fig. 11).

Example 14: hZAC expression induced a G1 arrest

mZAC, like p53, inhibits tumor cell growth through induction of apoptosis and cell cycle arrest, see Examples 5 to 7. In order to determine whether hZAC retrieved the same mechanisms for the control of cell proliferation the cell cycle progression of SaOs-2 cells upon transient expression of hZAC was first investigated. SaOs-2 cells were transiently transfected with different amounts of plasmid encoding mZAC, hZAC or p53, together with pRK5-CD20 encoding the CD20 antigen which

was used as a marker for selection of the transfected cells. Propidium-iodide staining was performed as previously described (Brons, Cytometry 11 (1990), 837-844). Cell cycle distribution was determined with a FACScan flow cytometer (Beckton-Dickinson). 5000 events corresponding to the 5% CD20 most positive cells were analysed using Modfit software (Verity Software House, Inc). hZAC induced a G1 arrest with increased proportion of cells in G0-G1 and decreased in S (Fig. 12A). mZAC induced a G1 block (Fig. 12A). p53-transiently transfected SaOs-2 cells were strongly arrested in G1 (Fig. 12A) as previously reported (Chen, Genes Develop. 10 (1996), 2438-2451).

mZAC, hZAC and p53 expression levels were evaluated by Western blots performed with the same anti-HA antibody. Western blots were performed on total cell lysates (20µg protein) using anti-HA antibody (clone 12CA5, Boehringer Mannheim, France) and peroxydase-linked anti-mouse Ig (Amersham, France). p53 and mZAC reached comparable high levels of expression (Fig. 12B). Transfection of higher amounts of plasmid was required to detect hZAC (Fig. 12B). However even non-detectable levels of hZAC could induce a G1 arrest (see pRK-hZAC 125 and 250 ng, Fig. 12A, 12B).

Example 15: hZAC expression induced apoptosis

Apoptosis was investigated by measuring genomic DNA laddering. Soluble DNA was prepared as previously described (Hockenberry, Nature 348 (1990), 334-336) and fractionated on a 1.2% agarose gel.

Mock-transfected (lane 1) or control (pRK5-CAT) transfected cells (lane 2) did not show any sign of apoptosis (Fig. 13). hZAC expression induced apoptosis of SaOs-2 transfected cells (lanes 6,7,8) as noted for mZAC (lanes 3,4,5) and p53 (lane 9) as described in Example 6.

In summary, we have demonstrated that mZAC (Bop1) establishes a new class of TSG with its mammalian counterparts, e.g. rat and, in particular, human (hZAC). The identity of mZAC and hZAC genes was emphasized by the identical in vitro

properties of both proteins, for example the antiproliferative properties of these nuclear transcriptional activators. hZAC is the only human gene so far beside p53 able to regulate both apoptosis and cell cycle. Since these pathways are known to be central to the activity of the TSG p53 (Bates, Curr. Opin. Genet. Dev. 6 (1996), 12-19) and since hZAC maps to 6q24-q25, a chromosomal region frequently lost in human tumors, it is conclusive that hZAC is a candidate for a human TSG.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any nucleic acid molecules, proteins, constructs or antibodies which are functionally equivalent are within the scope of this invention. Indeed, various modification of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Max-Planck-Gesellschaft zur Foerderung der Wissenschaften e.V.
(B) STREET: none
(C) CITY: Berlin
(E) COUNTRY: DE
(F) POSTAL CODE (ZIP): none

(A) NAME: CNRS
(B) STREET: rue de la cardonille
(C) CITY: Montpellier Cedex 05
(E) COUNTRY: FR
(F) POSTAL CODE (ZIP): 34094

(ii) TITLE OF INVENTION: Nucleic acid molecules coding for mammalian tumor suppressor proteins and methods for their isolation

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2790 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:542..2545

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTCGGGA GAGCAAGCGG GCATCTCCTG GGCGCCGTCA TGGCTGCTTA GGCTGCGCTG 60

CCTGCGGATC GCGGATCCGG GATCGGAGAT CTGACGGCGA CGCCTGAGTC CGGCTAGGGT 120

AGGTCTGGGT TGGAGTCTGT GCCTGCTTCC TTGGCGTGTG GTTGTTCCTG CTTGATTGCT 180

TCAGCGTGCC ATCGGCTTCG TATTTGCATA GGAGTCAGAG GAGTTAATCT TGTCTCCTCG 240

AAGATAGACT CTCATGGTTT ATGATCCATC TCTGTGAGAA GACTTTATTT GTCTGTCTCT 300

```
TCTCACAGGT TTGAGTCTTC AGACTTCTAC AGAACTCCAT AATATCTGCC TCACAGCTGG      360

CTTTCCTGCT CTCACAGAAG ATACCCAGCT ATTGTGCTCT GGATCTCTCC TGGCTGCTAG      420

GCTGTAGCGC TGCCTTTCTG GAGTCAGGCT GTAGTGACTC CCCACCTTCT TTCTGTCTGG      480

GCTTAAATGG CACAGCAGTT CCTCAGCACA TCTGAAGAAG AAAGTGTGAG AACCAAAGGC      540

C ATG GCT CCA TTC CGC TGT CAA AAA TGT GGC AAG TCC TTC GTC ACC          586
  Met Ala Pro Phe Arg Cys Gln Lys Cys Gly Lys Ser Phe Val Thr
    1               5                  10                  15

CTG GAG AAG TTC ACC ATT CAC AAT TAT TCC CAC TCC AGG GAG CGC CCA        634
Leu Glu Lys Phe Thr Ile His Asn Tyr Ser His Ser Arg Glu Arg Pro
                 20                  25                  30

TTC AAG TGC TCG AAG GCT GAG TGT GGC AAA GCC TTC GTC TCC AAG TAT        682
Phe Lys Cys Ser Lys Ala Glu Cys Gly Lys Ala Phe Val Ser Lys Tyr
             35                  40                  45

AAG CTG ATG AGA CAC ATG GCC ACA CAC TCG CCA CAG AAG ATT CAC CAG        730
Lys Leu Met Arg His Met Ala Thr His Ser Pro Gln Lys Ile His Gln
         50                  55                  60

TGT ACT CAC TGT GAG AAG ACA TTC AAC CGG AAG GAC CAC CTG AAG AAC        778
Cys Thr His Cys Glu Lys Thr Phe Asn Arg Lys Asp His Leu Lys Asn
     65                  70                  75

CAC CTC CAG ACC CAC GAT CCC AAC AAG ATC TCC TAC GCG TGT GAC GAT        826
His Leu Gln Thr His Asp Pro Asn Lys Ile Ser Tyr Ala Cys Asp Asp
 80                  85                  90                  95

TGC GGC AAG AAG TAC CAC ACC ATG CTG GGC TAC AAG AGG CAC CTG GCC        874
Cys Gly Lys Lys Tyr His Thr Met Leu Gly Tyr Lys Arg His Leu Ala
                100                 105                 110

CTG CAC TCG GCG AGC AAT GGC GAT CTC ACC TGT GGG GTG TGC ACC CTG        922
Leu His Ser Ala Ser Asn Gly Asp Leu Thr Cys Gly Val Cys Thr Leu
            115                 120                 125

GAG CTG GGG AGC ACC GAG GTC CTG CTG GAC CAC CTC AAG TCT CAC GCG        970
Glu Leu Gly Ser Thr Glu Val Leu Leu Asp His Leu Lys Ser His Ala
        130                 135                 140

GAA GAA AAG GCC AAC CAG GCA CCC AGG GAG AAG AAA TAC CAG TGC GAC       1018
Glu Glu Lys Ala Asn Gln Ala Pro Arg Glu Lys Lys Tyr Gln Cys Asp
    145                 150                 155

CAC TGT GAT AGA TGC TTC TAC ACC CGG AAA GAT GTG CGT CGC CAC CTG       1066
His Cys Asp Arg Cys Phe Tyr Thr Arg Lys Asp Val Arg Arg His Leu
160                 165                 170                 175

GTG GTC CAC ACA GGA TGC AAG GAC TTC CTG TGT CAG TTC TGT GCC CAG       1114
Val Val His Thr Gly Cys Lys Asp Phe Leu Cys Gln Phe Cys Ala Gln
                180                 185                 190

AGA TTT GGG CGC AAA GAC CAC CTC ACT CGT CAC ACC AAG AAG ACC CAC       1162
Arg Phe Gly Arg Lys Asp His Leu Thr Arg His Thr Lys Lys Thr His
            195                 200                 205
```

```
TCC CAG GAG CTG ATG CAA GAG AAT ATG CAG GCA GGA GAT TAC CAG AGC      1210
Ser Gln Glu Leu Met Gln Glu Asn Met Gln Ala Gly Asp Tyr Gln Ser
        210                 215                 220

AAT TTC CAA CTC ATT GCG CCT TCA ACT TCG TTC CAG ATA AAG GTT GAT      1258
Asn Phe Gln Leu Ile Ala Pro Ser Thr Ser Phe Gln Ile Lys Val Asp
    225                 230                 235

CCC ATG CCT CCT TTC CAG CTA GGA GCG GCT CCC GAG AAC GGG CTT GAT      1306
Pro Met Pro Pro Phe Gln Leu Gly Ala Ala Pro Glu Asn Gly Leu Asp
240                 245                 250                 255

GGT GGC TTG CCA CCC GAG GTT CAT GGT CTA GTG CTT GCT GCC CCA GAA      1354
Gly Gly Leu Pro Pro Glu Val His Gly Leu Val Leu Ala Ala Pro Glu
                260                 265                 270

GAA GCT CCC CAA CCC ATG CCG CCC TTG GAG CCT TTG GAG CCT TTG GAG      1402
Glu Ala Pro Gln Pro Met Pro Pro Leu Glu Pro Leu Glu Pro Leu Glu
            275                 280                 285

CCT TTG GAG CCT TTG GAG CCG ATG CAG TCT TTG GAG CCT TTG CAG CCT      1450
Pro Leu Glu Pro Leu Glu Pro Met Gln Ser Leu Glu Pro Leu Gln Pro
        290                 295                 300

TTG GAG CCG ATG CAG CCT TTG GAG CCA ATG CAG CCT TTG GAG CCG ATG      1498
Leu Glu Pro Met Gln Pro Leu Glu Pro Met Gln Pro Leu Glu Pro Met
    305                 310                 315

CAG CCT TTA GAG CCT TTG GAG CCT CTG GAG CCG ATG CAG CCT TTG GAG      1546
Gln Pro Leu Glu Pro Leu Glu Pro Leu Glu Pro Met Gln Pro Leu Glu
320                 325                 330                 335

CCG ATG CAG CCT TTG GAG CCT ATG CAG CCA ATG CTG CCA ATG CAG CCA      1594
Pro Met Gln Pro Leu Glu Pro Met Gln Pro Met Leu Pro Met Gln Pro
                340                 345                 350

ATG CAG CCA ATG CAG CCA ATG CAG CCA ATG CTG CCA ATG CAG CCA ATG      1642
Met Gln Pro Met Gln Pro Met Gln Pro Met Leu Pro Met Gln Pro Met
            355                 360                 365

CTG CCA ATG CAG CCA ATG CAG CCA ATG CAG CCA ATG CTG CCA ATG CCA      1690
Leu Pro Met Gln Pro Met Gln Pro Met Gln Pro Met Leu Pro Met Pro
        370                 375                 380

GAG CCG TCT TTC ACT CTG CAC CCT GGC GTA GTT CCC ACC TCT CCT CCC      1738
Glu Pro Ser Phe Thr Leu His Pro Gly Val Val Pro Thr Ser Pro Pro
    385                 390                 395

CCA ATT ATT CTT CAG GAG CAT AAG TAT AAT CCT GTT CCT ACC TCA TAT      1786
Pro Ile Ile Leu Gln Glu His Lys Tyr Asn Pro Val Pro Thr Ser Tyr
400                 405                 410                 415

GCC CCA TTT GTA GGC ATG CCC GTC AAA GCA GAT GGC AAG GCC TTT TGC      1834
Ala Pro Phe Val Gly Met Pro Val Lys Ala Asp Gly Lys Ala Phe Cys
                420                 425                 430

AAC GTG GGT TTC TTT GAG GAA TTT CCT CTG CAA GAG CCT CAG GCG CCT      1882
Asn Val Gly Phe Phe Glu Glu Phe Pro Leu Gln Glu Pro Gln Ala Pro
```

435 440 445

CTC AAG TTC AAC CCA TGT TTT GAG ATG CCT ATG GAG GGG TTT GGG AAA 1930
Leu Lys Phe Asn Pro Cys Phe Glu Met Pro Met Glu Gly Phe Gly Lys
450 455 460

GTC ACC CTG TCC AAA GAG CTG CTG GTA GAT GCT GTG AAT ATA GCC ATT 1978
Val Thr Leu Ser Lys Glu Leu Leu Val Asp Ala Val Asn Ile Ala Ile
465 470 475

CCT GCC TCT CTG GAG ATT TCC TCC CTA TTG GGG TTT TGG CAG CTC CCC 2026
Pro Ala Ser Leu Glu Ile Ser Ser Leu Leu Gly Phe Trp Gln Leu Pro
480 485 490 495

CCT CCT ACT CCC CAG AAT GGC TTT GTG AAT AGC ACC ATC CCT GTG GGG 2074
Pro Pro Thr Pro Gln Asn Gly Phe Val Asn Ser Thr Ile Pro Val Gly
500 505 510

CCT GGG GAG CCA CTG CCC CAT AGG ATA ACC TGT CTG GCG CAG CAG CAG 2122
Pro Gly Glu Pro Leu Pro His Arg Ile Thr Cys Leu Ala Gln Gln Gln
515 520 525

CCA CCG CCA CTG CCG CCG CCA CCA CCG CTG CCA CTG CCA CAG CCA CTG 2170
Pro Pro Pro Leu Pro Pro Pro Pro Pro Leu Pro Leu Pro Gln Pro Leu
530 535 540

CCA GTG CCA CAG CCA CTA CCA CAG CCA CAG ATG CAG CCA CAG TTT CAG 2218
Pro Val Pro Gln Pro Leu Pro Gln Pro Gln Met Gln Pro Gln Phe Gln
545 550 555

TTG CAG ATC CAG CCC CAG ATG CAG CTA CCA CAG CTG CTG CCG CAA CTG 2266
Leu Gln Ile Gln Pro Gln Met Gln Leu Pro Gln Leu Leu Pro Gln Leu
560 565 570 575

CAA CCT CAG CAG CAG CCT GAT CCT GAG CCA GAG CCA GAG CCA GAG CCA 2314
Gln Pro Gln Gln Gln Pro Asp Pro Glu Pro Glu Pro Glu Pro Glu Pro
580 585 590

GAG CCA GAG CCA GAG CCA GAG CCG GAA CCG GAA CCG GAG CCA GAG CCA 2362
Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro
595 600 605

GAG CCA GAA CCA GAG CCA GAG GAA GAA CAG GAA GAG GCA GAA GAA GAG 2410
Glu Pro Glu Pro Glu Pro Glu Glu Glu Gln Glu Glu Ala Glu Glu Glu
610 615 620

GCA GAG GAA GGA GCA GAG GAA GGA GCA GAA CCA GAG GCA CAG GCA GAA 2458
Ala Glu Glu Gly Ala Glu Glu Gly Ala Glu Pro Glu Ala Gln Ala Glu
625 630 635

GAA GAG GAA GAG GAA GAG GAA GCG GAA GAG CCA CAG CCA GAA GAA GCC 2506
Glu Glu Glu Glu Glu Glu Glu Ala Glu Glu Pro Gln Pro Glu Glu Ala
640 645 650 655

CAA ATA GCA GGA CTC GTC TAT AAG AAA TGG ACA GTT TAG TTCCTCTTCT 2555
Gln Ile Ala Gly Leu Val Tyr Lys Lys Trp Thr Val *
660 665

TGTTAGCTTA CTCTGTAGTT TCTTCTTCTT GTTGCCCATT GTGTAGCTTT ATAGAGTGTG 2615

ACGCTATTGA TGTCTCCATT TTTTAAAGTG AATTTAAATG TACTGTTCAA TATTTTTCAT 2675

GTGATGTTGT TCCAATGTGA GTTACGACTT CATTTATCTT AAAGACAAAA CTGGTTGTCA 2735

GTCATATCTG ACAGAAGAAA GAAATCACTG TGTAACCAAG CCATATAGCG GCCGC 2790

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 668 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Ala Pro Phe Arg Cys Gln Lys Cys Gly Lys Ser Phe Val Thr Leu
1               5                   10                  15

Glu Lys Phe Thr Ile His Asn Tyr Ser His Ser Arg Glu Arg Pro Phe
            20                  25                  30

Lys Cys Ser Lys Ala Glu Cys Gly Lys Ala Phe Val Ser Lys Tyr Lys
        35                  40                  45

Leu Met Arg His Met Ala Thr His Ser Pro Gln Lys Ile His Gln Cys
    50                  55                  60

Thr His Cys Glu Lys Thr Phe Asn Arg Lys Asp His Leu Lys Asn His
65                  70                  75                  80

Leu Gln Thr His Asp Pro Asn Lys Ile Ser Tyr Ala Cys Asp Asp Cys
                85                  90                  95

Gly Lys Lys Tyr His Thr Met Leu Gly Tyr Lys Arg His Leu Ala Leu
            100                 105                 110

His Ser Ala Ser Asn Gly Asp Leu Thr Cys Gly Val Cys Thr Leu Glu
        115                 120                 125

Leu Gly Ser Thr Glu Val Leu Leu Asp His Leu Lys Ser His Ala Glu
    130                 135                 140

Glu Lys Ala Asn Gln Ala Pro Arg Glu Lys Lys Tyr Gln Cys Asp His
145                 150                 155                 160

Cys Asp Arg Cys Phe Tyr Thr Arg Lys Asp Val Arg Arg His Leu Val
                165                 170                 175

Val His Thr Gly Cys Lys Asp Phe Leu Cys Gln Phe Cys Ala Gln Arg
            180                 185                 190

Phe Gly Arg Lys Asp His Leu Thr Arg His Thr Lys Lys Thr His Ser
        195                 200                 205

Gln Glu Leu Met Gln Glu Asn Met Gln Ala Gly Asp Tyr Gln Ser Asn
```

210 215 220

Phe Gln Leu Ile Ala Pro Ser Thr Ser Phe Gln Ile Lys Val Asp Pro
225 230 235 240

Met Pro Pro Phe Gln Leu Gly Ala Ala Pro Glu Asn Gly Leu Asp Gly
245 250 255

Gly Leu Pro Pro Glu Val His Gly Leu Val Leu Ala Ala Pro Glu Glu
260 265 270

Ala Pro Gln Pro Met Pro Pro Leu Glu Pro Leu Glu Pro Leu Glu Pro
275 280 285

Leu Glu Pro Leu Glu Pro Met Gln Ser Leu Glu Pro Leu Gln Pro Leu
290 295 300

Glu Pro Met Gln Pro Leu Glu Pro Met Gln Pro Leu Glu Pro Met Gln
305 310 315 320

Pro Leu Glu Pro Leu Glu Pro Leu Glu Pro Met Gln Pro Leu Glu Pro
325 330 335

Met Gln Pro Leu Glu Pro Met Gln Pro Met Leu Pro Met Gln Pro Met
340 345 350

Gln Pro Met Gln Pro Met Gln Pro Met Leu Pro Met Gln Pro Met Leu
355 360 365

Pro Met Gln Pro Met Gln Pro Met Gln Pro Met Leu Pro Met Pro Glu
370 375 380

Pro Ser Phe Thr Leu His Pro Gly Val Val Pro Thr Ser Pro Pro Pro
385 390 395 400

Ile Ile Leu Gln Glu His Lys Tyr Asn Pro Val Pro Thr Ser Tyr Ala
405 410 415

Pro Phe Val Gly Met Pro Val Lys Ala Asp Gly Lys Ala Phe Cys Asn
420 425 430

Val Gly Phe Phe Glu Glu Phe Pro Leu Gln Glu Pro Gln Ala Pro Leu
435 440 445

Lys Phe Asn Pro Cys Phe Glu Met Pro Met Glu Gly Phe Gly Lys Val
450 455 460

Thr Leu Ser Lys Glu Leu Leu Val Asp Ala Val Asn Ile Ala Ile Pro
465 470 475 480

Ala Ser Leu Glu Ile Ser Ser Leu Leu Gly Phe Trp Gln Leu Pro Pro
485 490 495

Pro Thr Pro Gln Asn Gly Phe Val Asn Ser Thr Ile Pro Val Gly Pro
500 505 510

Gly Glu Pro Leu Pro His Arg Ile Thr Cys Leu Ala Gln Gln Gln Pro
515 520 525

```
Pro Pro Leu Pro Pro Pro Pro Pro Leu Pro Leu Pro Gln Pro Leu Pro
    530                 535                 540

Val Pro Gln Pro Leu Pro Gln Pro Gln Met Gln Pro Gln Phe Gln Leu
545                 550                 555                 560

Gln Ile Gln Pro Gln Met Gln Leu Pro Gln Leu Leu Pro Gln Leu Gln
                565                 570                 575

Pro Gln Gln Gln Pro Asp Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu
            580                 585                 590

Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu
        595                 600                 605

Pro Glu Pro Glu Pro Glu Glu Glu Gln Glu Glu Ala Glu Glu Glu Ala
    610                 615                 620

Glu Glu Gly Ala Glu Glu Gly Ala Glu Pro Glu Ala Gln Ala Glu Glu
625                 630                 635                 640

Glu Glu Glu Glu Glu Glu Ala Glu Glu Pro Gln Pro Glu Glu Ala Gln
                645                 650                 655

Ile Ala Gly Leu Val Tyr Lys Lys Trp Thr Val  *
            660                 665
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
- (A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGTCTCGAG GCCTTTGCGG CCGCTATANN NNNNNN 36

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGGCCATGG 9

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:3
(D) OTHER INFORMATION:/note= "N at position 3 is A or G."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCNCCATGG 9

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

His Ser Arg Glu Arg Pro Phe Lys Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(ix) FEATURE:
(A) NAME/KEY: Cross-links
(B) LOCATION:2
(D) OTHER INFORMATION:/note= "X at position 2 is S or T."

(ix) FEATURE:
(A) NAME/KEY: Cross-links
(B) LOCATION:7
(D) OTHER INFORMATION:/note= "X at position 7 is F or Y."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
His Xaa Gly Glu Lys Pro Xaa Xaa Cys
1               5
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
His Ser Pro Gln Lys
1               5
```

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
Lys Lys Trp Thr
1
```

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GTGATGGCGG CCGCCATTCC GCTGTCAAAA ATGTG 35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCGCGCCTCG AGGGTCTTCT TGGTGTGACG 30

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCGGCCGCAG AGCCGTCTTT CACTC 25

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CCGCGCCTCG AGAACTGTCC ATTTCTTATA GAC 33

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATAGCAGTGA GTGCTGTG 18

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GTTTCTTTTC AGGGACTC 18

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2334 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:803..2192

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CGGTTCTTTC AATTCAGAAT TTGTTTTAGG TTCTGTTATT GCATAGATTT GCATACCTGT 60

TTTATGGTAT TTTAATACTG TTGGTTTTAA AAAATACCAT TTCCTCTGAG TGCTGTTCTG 120

AATATATTAT GTAAGCAATT TTGTGTGTTC TTTTTTTTCC ACTTGCATAA AGCAGGGGAA 180

AAGTTGAGAG TTTTTCTTAA TCCAGTCCCA AGTAGGACAA AGGATATGAG TGTTTAAAGA 240

TCATCTATTA AAATGCATGA AAAAACACTA GAAAATCTCC TGTGCACATC GCCAGTCGTG 300

TGTGTGCTCT AGAAGTGAAG TTCAGGGGGT AACATAATGG AGGAATGTTT TCCTAGCTTC 360

ATTCCCTGAC GATGTACAAG GTCTCTTCTC ACAGGTTTGA ATCTTCAGAC AAACTTCTGG 420

```
GAGGACTGGG AGGACTCGGT CCCTGCCTCG CAGCAGATGT TCCCTGTCAC TCAGTAGCCA     480

ATCCGGGGGA CCCAGGACAT GCCCCAGCTA TAGTGATGCA GATTACCTTT CTGGTCCTGA     540

ATCGCACCTG TGCCTCGAGA CTTTCTCCCC TCAGCTTGAG ACTGCATGTA AACTGGGATG     600

TGTGAAAGCA GGAAGCAAAG CTAGTGACAG CTGAGAGGTC CATGTCTGGG TAGAACCAGG     660

CCCACGATGC TGCCTCTCCC GTGGTCTGGA GTTCAGCTGC AGGGACTCTG CTGATTGGCC     720

CAGCACCATC GTTCTGTTTG TGCTTAAATG GCACAGCATT TGGTCAGCAC ATCTGAAAAG     780

GAAGGTGTGA GAAGCAAAGC CC ATG GCC ACG TTC CCC TGC CAG TTA TGT GGC      832
                         Met Ala Thr Phe Pro Cys Gln Leu Cys Gly
                           1               5                  10

AAG ACG TTC CTC ACC CTG GAG AAG TTC ACG ATT CAC AAT TAT TCC CAC       880
Lys Thr Phe Leu Thr Leu Glu Lys Phe Thr Ile His Asn Tyr Ser His
                 15                  20                  25

TCC AGG GAG CGG CCG TAC AAG TGT GTG CAG CCT GAC TGT GGC AAA GCC       928
Ser Arg Glu Arg Pro Tyr Lys Cys Val Gln Pro Asp Cys Gly Lys Ala
             30                  35                  40

TTT GTT TCC AGA TAT AAA TTG ATG AGG CAT ATG GCT ACC CAT TCT CCC       976
Phe Val Ser Arg Tyr Lys Leu Met Arg His Met Ala Thr His Ser Pro
         45                  50                  55

CAG AAA TCT CAC CAG TGT GCT CAC TGT GAG AAG ACG TTC AAC CGG AAA      1024
Gln Lys Ser His Gln Cys Ala His Cys Glu Lys Thr Phe Asn Arg Lys
     60                  65                  70

GAC CAC CTG AAA AAC CAC CTC CAG ACC CAC GAC CCC AAC AAA ATG GCC      1072
Asp His Leu Lys Asn His Leu Gln Thr His Asp Pro Asn Lys Met Ala
 75                  80                  85                  90

TTT GGG TGT GAG GAG TGT GGG AAG AAG TAC AAC ACC ATG CTG GGC TAT      1120
Phe Gly Cys Glu Glu Cys Gly Lys Lys Tyr Asn Thr Met Leu Gly Tyr
                 95                 100                 105

AAG AGG CAC CTG GCC CTC CAT GCG GCC AGC AGT GGG GAC CTC ACC TGT      1168
Lys Arg His Leu Ala Leu His Ala Ala Ser Ser Gly Asp Leu Thr Cys
            110                 115                 120

GGG GTC TGT GCC CTG GAG CTA GGG AGC ACC GAG GTG CTA CTG GAC CAC      1216
Gly Val Cys Ala Leu Glu Leu Gly Ser Thr Glu Val Leu Leu Asp His
        125                 130                 135

CTC AAA GCC CAT GCG GAA GAG AAG CCC CCT AGC GGA ACC AAG GAA AAG      1264
Leu Lys Ala His Ala Glu Glu Lys Pro Pro Ser Gly Thr Lys Glu Lys
    140                 145                 150

AAG CAC CAG TGC GAC CAC TGT GAA AGA TGC TTC TAC ACC CGG AAG GAT      1312
Lys His Gln Cys Asp His Cys Glu Arg Cys Phe Tyr Thr Arg Lys Asp
155                 160                 165                 170
```

```
GTG CGA CGC CAC CTG GTG GTC CAC ACA GGA TGC AAG GAC TTC CTG TGC      1360
Val Arg Arg His Leu Val Val His Thr Gly Cys Lys Asp Phe Leu Cys
                175                 180                 185

CAG TTC TGT GCC CAG AGA TTT GGG CGC AAG GAT CAC CTC ACC CGG CAT      1408
Gln Phe Cys Ala Gln Arg Phe Gly Arg Lys Asp His Leu Thr Arg His
            190                 195                 200

ACC AAG AAG ACC CAC TCA CAG GAG CTG ATG AAA GAG AGC TTG CAG ACC      1456
Thr Lys Lys Thr His Ser Gln Glu Leu Met Lys Glu Ser Leu Gln Thr
        205                 210                 215

GGA GAC CTT CTG AGC ACC TTC CAC ACC ATC TCG CCT TCA TTC CAA CTG      1504
Gly Asp Leu Leu Ser Thr Phe His Thr Ile Ser Pro Ser Phe Gln Leu
    220                 225                 230

AAG GCT GCT GCC TTG CCT CCT TTC CCT TTA GGA GCT TCT GCC CAG AAC      1552
Lys Ala Ala Ala Leu Pro Pro Phe Pro Leu Gly Ala Ser Ala Gln Asn
235                 240                 245                 250

GGG CTT GCA AGT AGC TTG CCA GCT GAG GTC CAT AGC CTC ACC CTC AGT      1600
Gly Leu Ala Ser Ser Leu Pro Ala Glu Val His Ser Leu Thr Leu Ser
                255                 260                 265

CCC CCA GAA CAA GCC GCC CAG CCT ATG CAG CCG CTG CCA GAG TCC CTG      1648
Pro Pro Glu Gln Ala Ala Gln Pro Met Gln Pro Leu Pro Glu Ser Leu
            270                 275                 280

GCC TCC CTC CAC CCC TCG GTA TCC CCT GGC TCT CCT CCG CCA CCC CTT      1696
Ala Ser Leu His Pro Ser Val Ser Pro Gly Ser Pro Pro Pro Pro Leu
        285                 290                 295

CCC AAT CAC AAG TAC AAC ACC ACT TCT ACC TCA TAC TCC CCA CTT GCA      1744
Pro Asn His Lys Tyr Asn Thr Thr Ser Thr Ser Tyr Ser Pro Leu Ala
    300                 305                 310

AGC CTG CCC CTC AAA GCA GAT ACT AAA GGT TTT TGC AAT ATC AGT TTG      1792
Ser Leu Pro Leu Lys Ala Asp Thr Lys Gly Phe Cys Asn Ile Ser Leu
315                 320                 325                 330

TTT GAG GAC TTG CCT CTG CAA GAG CCT CAG TCA CCT CAA AAG CTC AAC      1840
Phe Glu Asp Leu Pro Leu Gln Glu Pro Gln Ser Pro Gln Lys Leu Asn
                335                 340                 345

CCA GGT TTT GAT CTG GCT AAG GGA AAT GCT GGT AAA GTA AAC CTG CCC      1888
Pro Gly Phe Asp Leu Ala Lys Gly Asn Ala Gly Lys Val Asn Leu Pro
            350                 355                 360

AAG GAG CTG CCT GCA GAT GCT GTG AAC CTA ACA ATA CCT GCC TCT CTG      1936
Lys Glu Leu Pro Ala Asp Ala Val Asn Leu Thr Ile Pro Ala Ser Leu
        365                 370                 375

GAC CTG TCC CCC CTG TTG GGC TTC TGG CAG CTG CCC CCT CCT GCT ACC      1984
Asp Leu Ser Pro Leu Leu Gly Phe Trp Gln Leu Pro Pro Pro Ala Thr
```

```
    380                 385                 390

CAA AAT ACC TTT GGG AAT AGC ACT CTT GCC CTG GGG CCT GGG GAA TCT      2032
Gln Asn Thr Phe Gly Asn Ser Thr Leu Ala Leu Gly Pro Gly Glu Ser
395                 400                 405                 410

TTG CCC CAC AGG TTA AGC TGT CTG GGG CAG CAG CAG CAA GAA CCC CCA      2080
Leu Pro His Arg Leu Ser Cys Leu Gly Gln Gln Gln Gln Glu Pro Pro
                415                 420                 425

CTT GCC ATG GGC ACT GTG AGC CTG GGC CAG CTC CCC CTG CCC CCC ATC      2128
Leu Ala Met Gly Thr Val Ser Leu Gly Gln Leu Pro Leu Pro Pro Ile
            430                 435                 440

CCT CAT GTG TTC TCA GCT GGC ACT GGC TCT GCC ATC CTG CCT CAT TTC      2176
Pro His Val Phe Ser Ala Gly Thr Gly Ser Ala Ile Leu Pro His Phe
        445                 450                 455

CAT CAT GCA TTC AGA T AATTGATTTT TAAAGTGTAT TTTTCGTATT              2222
His His Ala Phe Arg
    460

CTGGAAGATG TTTTAAGAAG CATTTTAAAT GTCAGTTACA ATATGAGAAA GATTTGGAAA    2282

ACGAGACTGG GACTATGGCT TATTCAGTGA TGACTGGCTT GAGATGATAA GA            2334
```

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 463 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
Met Ala Thr Phe Pro Cys Gln Leu Cys Gly Lys Thr Phe Leu Thr Leu
  1               5                  10                  15

Glu Lys Phe Thr Ile His Asn Tyr Ser His Ser Arg Glu Arg Pro Tyr
             20                  25                  30

Lys Cys Val Gln Pro Asp Cys Gly Lys Ala Phe Val Ser Arg Tyr Lys
         35                  40                  45

Leu Met Arg His Met Ala Thr His Ser Pro Gln Lys Ser His Gln Cys
     50                  55                  60

Ala His Cys Glu Lys Thr Phe Asn Arg Lys Asp His Leu Lys Asn His
 65                  70                  75                  80

Leu Gln Thr His Asp Pro Asn Lys Met Ala Phe Gly Cys Glu Glu Cys
                 85                  90                  95
```

```
Gly Lys Lys Tyr Asn Thr Met Leu Gly Tyr Lys Arg His Leu Ala Leu
            100                 105                 110

His Ala Ala Ser Ser Gly Asp Leu Thr Cys Gly Val Cys Ala Leu Glu
        115                 120                 125

Leu Gly Ser Thr Glu Val Leu Leu Asp His Leu Lys Ala His Ala Glu
    130                 135                 140

Glu Lys Pro Pro Ser Gly Thr Lys Glu Lys Lys His Gln Cys Asp His
145                 150                 155                 160

Cys Glu Arg Cys Phe Tyr Thr Arg Lys Asp Val Arg Arg His Leu Val
                165                 170                 175

Val His Thr Gly Cys Lys Asp Phe Leu Cys Gln Phe Cys Ala Gln Arg
            180                 185                 190

Phe Gly Arg Lys Asp His Leu Thr Arg His Thr Lys Lys Thr His Ser
        195                 200                 205

Gln Glu Leu Met Lys Glu Ser Leu Gln Thr Gly Asp Leu Leu Ser Thr
    210                 215                 220

Phe His Thr Ile Ser Pro Ser Phe Gln Leu Lys Ala Ala Ala Leu Pro
225                 230                 235                 240

Pro Phe Pro Leu Gly Ala Ser Ala Gln Asn Gly Leu Ala Ser Ser Leu
                245                 250                 255

Pro Ala Glu Val His Ser Leu Thr Leu Ser Pro Pro Glu Gln Ala Ala
            260                 265                 270

Gln Pro Met Gln Pro Leu Pro Glu Ser Leu Ala Ser Leu His Pro Ser
        275                 280                 285

Val Ser Pro Gly Ser Pro Pro Pro Pro Leu Pro Asn His Lys Tyr Asn
    290                 295                 300

Thr Thr Ser Thr Ser Tyr Ser Pro Leu Ala Ser Leu Pro Leu Lys Ala
305                 310                 315                 320

Asp Thr Lys Gly Phe Cys Asn Ile Ser Leu Phe Glu Asp Leu Pro Leu
                325                 330                 335

Gln Glu Pro Gln Ser Pro Gln Lys Leu Asn Pro Gly Phe Asp Leu Ala
            340                 345                 350

Lys Gly Asn Ala Gly Lys Val Asn Leu Pro Lys Glu Leu Pro Ala Asp
        355                 360                 365

Ala Val Asn Leu Thr Ile Pro Ala Ser Leu Asp Leu Ser Pro Leu Leu
    370                 375                 380
```

Gly Phe Trp Gln Leu Pro Pro Pro Ala Thr Gln Asn Thr Phe Gly Asn
385 390 395 400

Ser Thr Leu Ala Leu Gly Pro Gly Glu Ser Leu Pro His Arg Leu Ser
405 410 415

Cys Leu Gly Gln Gln Gln Gln Glu Pro Pro Leu Ala Met Gly Thr Val
420 425 430

Ser Leu Gly Gln Leu Pro Leu Pro Pro Ile Pro His Val Phe Ser Ala
435 440 445

Gly Thr Gly Ser Ala Ile Leu Pro His Phe His His Ala Phe Arg
450 455 460

BUDAPESTER VERTRAG ÜBER DIE INTERNATIONALE ANERKENNUNG DER HINTERLEGUNG VON MIKROORGANISMEN FÜR DIE ZWECKE VON PATENTVERFAHREN

INTERNATIONALES FORMBLATT

Prof. Dr. Dr. F. Holsboer
Max-Planck-Institut für
Psychiatrie-Klinisches Institut
Kraepelinstr. 10
80804 München

EMPFANGSBESTÄTIGUNG BEI ERSTHINTERLEGUNG,
ausgestellt gemäß Regel 7.1 von der unten angegebenen
INTERNATIONALEN HINTERLEGUNGSSTELLE

I. KENNZEICHNUNG DES MIKROORGANISMUS	
Vom HINTERLEGER zugeteiltes Bezugszeichen: pBluescript II SK(-)p2195 (NotI)	Von der INTERNATIONALEN HINTERLEGUNGSSTELLE zugeteilte EINGANGSNUMMER: DSM 11112

II. WISSENSCHAFTLICHE BESCHREIBUNG UND/ODER VORGESCHLAGENE TAXONOMISCHE BEZEICHNUNG

Mit dem unter I. bezeichneten Mikroorganismus wurde

(X) eine wissenschaftliche Beschreibung
( ) eine vorgeschlagene taxonomische Bezeichnung

eingereicht.
(Zutreffendes ankreuzen)

III. EINGANG UND ANNAHME

Diese internationale Hinterlegungsstelle nimmt den unter I bezeichneten Mikroorganismus an, der bei ihr am 1996-08-05 (Datum der Ersthinterlegung)[1] eingegangen ist.

IV. EINGANG DES ANTRAGS AUF UMWANDLUNG

Der unter I bezeichnete Mikroorganismus ist bei dieser Internationalen Hinterlegungsstelle am eingegangen (Datum der Ersthinterlegung) und ein Antrag auf Umwandlung dieser Ersthinterlegung in eine Hinterlegung gemäß Budapester Vertrag ist am eingegangen (Datum des Eingangs des Antrags auf Umwandlung).

V. INTERNATIONALE HINTERLEGUNGSSTELLE

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Anschrift: Mascheroder Weg 1b D-38124 Braunschweig	Unterschrift(en) der zur Vertretung der internationalen Hinterlegungsstelle befugten Person(en) oder des (der) von ihr ermächtigten Bediensteten: U. Weiss Datum: 1996-08-12

[1] Falls Regel 6.4 Buchstabe d zutrifft, ist dies der Zeitpunkt, zu dem der Status einer internationalen Hinterlegungsstelle erworben worden ist.

Formblatt DSMZ-BP/4 (einzige Seite) 0196

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Prof. Dr. Dr. F. Holsboer
Max-Planck-Institut für
Psychiatrie-Klinisches Institut
Kraepelinstr. 10
80804 München

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Prof. Dr. Dr. F. Holsboer Max-Planck-Institut für Address: Psychiatrie-Klinisches Institut Kraepelinstr. 10 80804 München	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 11112 Date of the deposit or the transfer[1]: 1996-08-06

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 1996-08-06 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Weis Date: 1996-08-12

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 0196

CLAIMS

1. A nucleic acid molecule encoding a protein having the biological activity of a tumor suppressor selected from the group consisting of:
   (a) nucleic acid molecules coding for a polypeptide comprising the amino acid sequence given in SEQ ID NO.2;
   (b) nucleic acid molecules comprising the nucleotide sequence given in SEQ ID NO.1;
   (c) nucleic acid molecules hybridizing to a nucleic acid molecule as defined in (a) or (b); and
   (d) nucleic acid molecules, the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of a nucleic acid molecule as defined in (a), (b) or (c).

2. A method for the identification and cloning of nucleic acid molecules encoding a protein having the biological activity of a tumor suppressor comprising the steps of:
   (i) transfecting mammalian cells with
      (a) a first vector comprising a scorable reporter gene operatively linked to regulatory elements comprising at least one cAMP responsive element so located relative to said reporter gene to permit cAMP inducible expression thereof; and
      (b) pools of expression vectors comprising nucleic acid molecules linked to regulatory elements allowing expression in the mammalian cells;
   (ii) cultivating the transfected cells under conditions which permit expression of the nucleic acid molecules present in the vectors;
   (iii) identifying those vector pools which lead after transfection to expression of said reporter gene in the mammalian cells;

(iv) optionally subdividing the vector pool(s) identified in step (iii) and repeating step (i) to (iii); and

(v) isolating from the so-identified vector pool(s) the nucleic acid molecule present in the vector(s) and testing its product for tumor suppressor activity.

3. The method of claim 2, wherein in step (ii) a ligand of a receptor which is capable of increasing the level of intracellular cAMP is added to the culture medium.

4. The method of claim 3, wherein the ligand is the peptide PACAP.

5. The method of any one of claims 2 to 4, wherein the mammalian cells are LLC-PK1 cells (ATCC CC101) or Saos-2 cells (ATCC HTB 85).

6. The method of any one of claims 2 to 5, wherein the cAMP responsive element is derived from a corticotropin releasing hormone gene.

7. The method of any one of claims 2 to 6, wherein the regulatory elements controlling the reporter gene are derived from MMTV.

8. The method of any one of claims 2 to 7, wherein the reporter gene codes for a luciferase.

9. The method of any one of claims 2 to 8, wherein the nucleic acid molecules present in the vectors of the vector pool are cDNAs.

10. The method of claim 9, wherein the cDNA is prepared from RNA obtained from mammalian, bacterial, fungal or plant cells or viruses.

11. A nucleic acid molecule obtainable by a method of any one of claims 2 to 10 which encodes a protein having tumor suppressor activity.

12. A nucleic acid molecule which hybridizes to a nucleic acid molecule of claim 1 or claim 11 and which encodes a mutated version of a protein as defined in claim 1 and 11 which has lost its tumor suppressor activity.

13. The nucleic acid molecule of claim 1, 11 or 12 which is DNA.

14. The nucleic acid molecule of claim 13 which is cDNA.

15. The nucleic acid molecule of claim 1 or of any one of claims 11 to 14, which is derived from a mammal.

16. The nucleic acid molecule of claim 15, wherein the mammal is human or mouse.

17. The nucleic acid molecule of claim 16, wherein the nucleic acid molecule comprises a nucleotide sequence encoding the amino acid sequence given in SEQ ID NO. 17 or the nucleotide sequence given in SEQ ID NO. 16.

18. A nucleic acid molecule of at least 15 nucleotides in length hybridizing specifically with a nucleic acid molecule of claim 1 or with a nucleic acid molecule of any one of claims 11 to 17 or to a complementary strand thereof.

19. A vector comprising a nucleic acid molecule of claim 1 or of any one of claims 11 to 17.

20. The vector of claim 19, wherein the nucleic acid molecule is operatively linked to regulatory elements permitting expression in prokaryotic and/or eukaryotic host cells.

21. A host cell comprising a vector of claim 19 or 20.

22. The host cell of claim 21, which is a bacterial, fungal, plant or animal cell.

23. The host cell of claim 22, which is a mammalian cell.

24. Method for the production of a polypeptide having the biological activity of a tumor suppressor comprising culturing a host cell of claim 22 or 23 under conditions allowing the expression of the polypeptide and recovering the produced polypeptide from the culture.

25. A polypeptide encoded by a nucleic acid molecule of claim 1 or of any one of claims 11 to 17 or produced by a method according to claim 24.

26. An antibody specifically recognizing a polypeptide of claim 25.

27. A pharmaceutical composition comprising a nucleic acid molecule of claim 1 or of any one of claims 11 to 17, or a nucleic acid molecule which is complementary to such a nucleic acid molecule, a nucleic acid molecule of claim 18, a vector of claim 19 or 20, a polypeptide of claim 25 and/or an antibody of claim 26, and optionally a pharmaceutically acceptable carrier.

28. A diagnostic composition comprising a nucleic acid molecule of claim 1, or of any one of claims 11 to 17 or a nucleic acid molecule which is complementary to such a nucleic acid molecule, a nucleic acid molecule

of claim 18, a vector of claim 19 or 20, a polypeptide of claim 25 and/or an antibody of claim 26, and optionally suitable means for detection.

29. A method for treating of a tumor comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.

30. A method for preventing of a tumor comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.

31. A method for delaying the reoccurrence of a tumor comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.

32. The method of 29, 30 or 31 wherein the tumor is benign or malign and most preferably derived from endocrine or neuronal tissues, i. e. breast, lung, colon, intestine, stomach, prostate, testis, ovary, thyroid, pancreas.

33. A method for treating of neuronal disorders comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.

34. A method for preventing neuronal disorders comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.

35. A method for delaying the reoccurrence of neuronal disorders comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.

36. A method for detecting expression of a tumor suppressor by detecting the presence of mRNA coding for a tumor suppressor which comprises

(a) obtaining mRNA from a cell;

(b) contacting the mRNA so obtained with a probe comprising a nucleic acid molecule of claim 18 under hybridizing conditions; and

(c) detecting the presence of mRNA hybridized to the probe and thereby detecting the expression of the tumor suppressor by the cell.

37. A method for detecting expression of a tumor suppressor by detecting the presence of a tumor suppressor which comprises:

(a) obtaining a cell sample from the subject;

(b) contacting the cell sample so obtained with an antibody of claim 26 under conditions permitting binding of the antibody to the tumor suppressor; and

(c) detecting the presence of the antibody so bound and thereby detecting the expression of the tumor suppressor.

38. The method of claim 37 for the detection of the expression of a tumor suppressor which has lost its tumor suppressor activity.

39. A method for diagnosing in a subject a predisposition to a tumor or to a disorder associated with the expression of a tumor suppressor allele which comprises:

(a) isolating DNA from victims of the tumor or the disorder;

(b) digesting the isolated DNA of step (a) with at least one restriction enzyme;

(c) electrophoretically separating the resulting DNA fragments on a sizing gel;

(d) contacting the resulting gel with a probe comprising a nucleic acid molecule of claim 18 labeled with a detectable marker;

(e) detecting labeled bands on a gel which have hybridized to the prob as defined in (d) to create a band pattern specific to the DNA of victims of the tumor or the disorder;

(f) preparing subject's DNA by steps (a) to (e) to produce detectable labeled bands on a gel; and

(g) comparing the band pattern specific to the DNA of victims of the tumor or the disorder of step (e) and the subject's DNA of step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the tumor or the disorder if the patterns are the same.

40. Use of an effective dose of a nucleic acid molecule of claim 1 or of any one of claims 11 to 17, or a nucleic acid molecule which is complementary to such a nucleic acid molecule for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.

41. Use of an effective dose of a nucleic acid molecule of claim 18 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.

42. Use of an effective dose of a vector of claim 19 or 20 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.

43. Use of an effective dose of a polypeptide of claim 25 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.

44. Use of an effective dose of an antibody of claim 26 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.

45. The use of any one of claims 40 to 44 wherein the disease is a tumor or a neuronal disorder.

46. The use of claim 45 wherein the tumor is benign or malign and most preferably derived from endocrine or neuronal tissues, i.e. breast, lung, colon, intestine, stomach, prostate, testis, ovary, thyroid, pancreas.

47. A process for identifying compounds effective as antagonists/inhibitors or agonists/activators to a tumor suppressor comprising:
   (a) contacting a cell which expresses the polypeptide of claim 25 with a compound to be screened; and
   (b) determining if the compound inhibits or enhances activation of the tumor suppressor.

48. An antagonist/inhibitor or agonist/activator to the polypeptide of claim 25 or identified according to the method of claim 47.

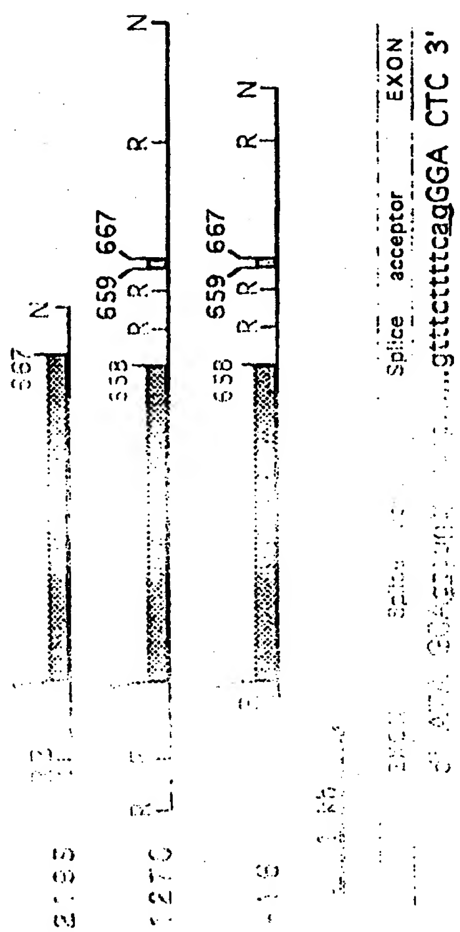
A

1	MAPFRCQKCG	KSFVTLEKFT	IHNYSHSRER
31	PFKCSKAECG	KAFVSKYKLM	RHMATHSPQK
61	IHQCTHCEKT	FNRKDHLKNH	LQTHDPNKIS
91	YACDDCGKKY	HTMLGYKRHL	ALHSASNGDL
121	TCGVCTLELG	STEVLLDHLK	SHAEEKANQA
151	PREKKYQCDH	CDRCFYTRKD	VRRHLVVHTG
181	CKDFLCQFCA	QRFGRKDHLT	RHTKKTHSQE
211	LMQENMQAGD	YQSNFQLIAP	STSFQIKVDP
241	MPPFQLGAAP	ENGLDGGLPP	EVHGLVLAAP
271	EEAPQPMPPL	EPLEPLEPLE	PLEPMQSLEP
301	LQPLEPMQPL	EPMQPLEPMQ	PLEPLEPLEP
331	MQPLEPMQPL	EPMQPMLPMQ	PMQPMQPMQP
361	MLPMQPMLPM	QPMQPMQPML	PMPEPSFTLH
391	PGVVPTSPPP	IILQEHKYNP	VPTSYAPFVG
421	MPVKADGKAF	CNVGFFEEFP	LQEPQAPLKF
451	NPCFEMPMEG	FGKVTLSKEL	LVDAVNIAIP
481	ASLEISSLLG	FWQLPPPTPQ	NGFVNSTIPV
511	GPGEPLPHRI	TCLAQQQPPP	LPPPPPLPLP
541	QPLPVPQPLP	QPQMQPQFQL	QIQPQMQLPQ
571	LLPQLQPQQQ	PDPEPEPEPE	PEPEPEPEPE
601	PEPEPEPEPE	PEPEEEQEEA	EEEAEEGAEE
631	GAEPEAQAEE	EEEEEEAEEP	QPEEAQIAGL
661	VYKKWTV		
	****		

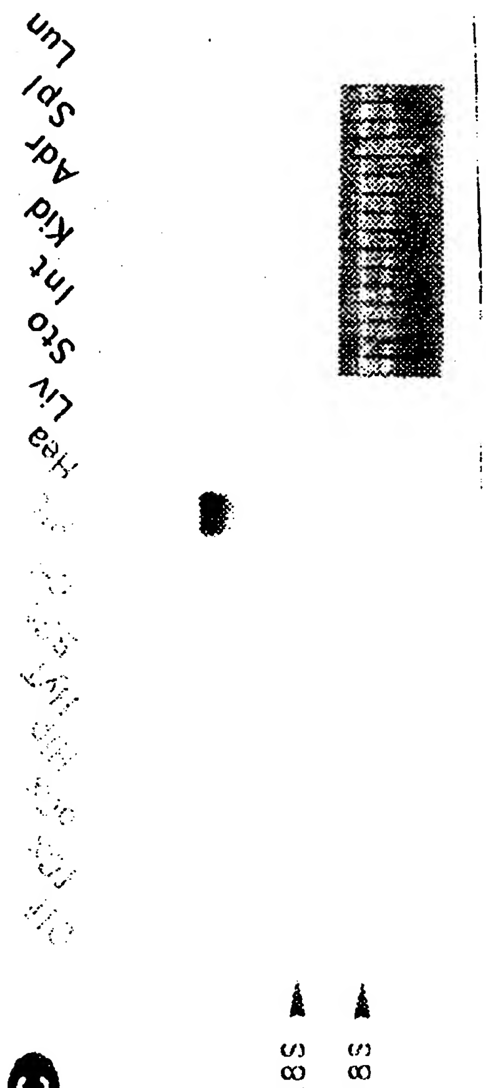
Figure 1 A

Figures 1 B and 1 C

B



C



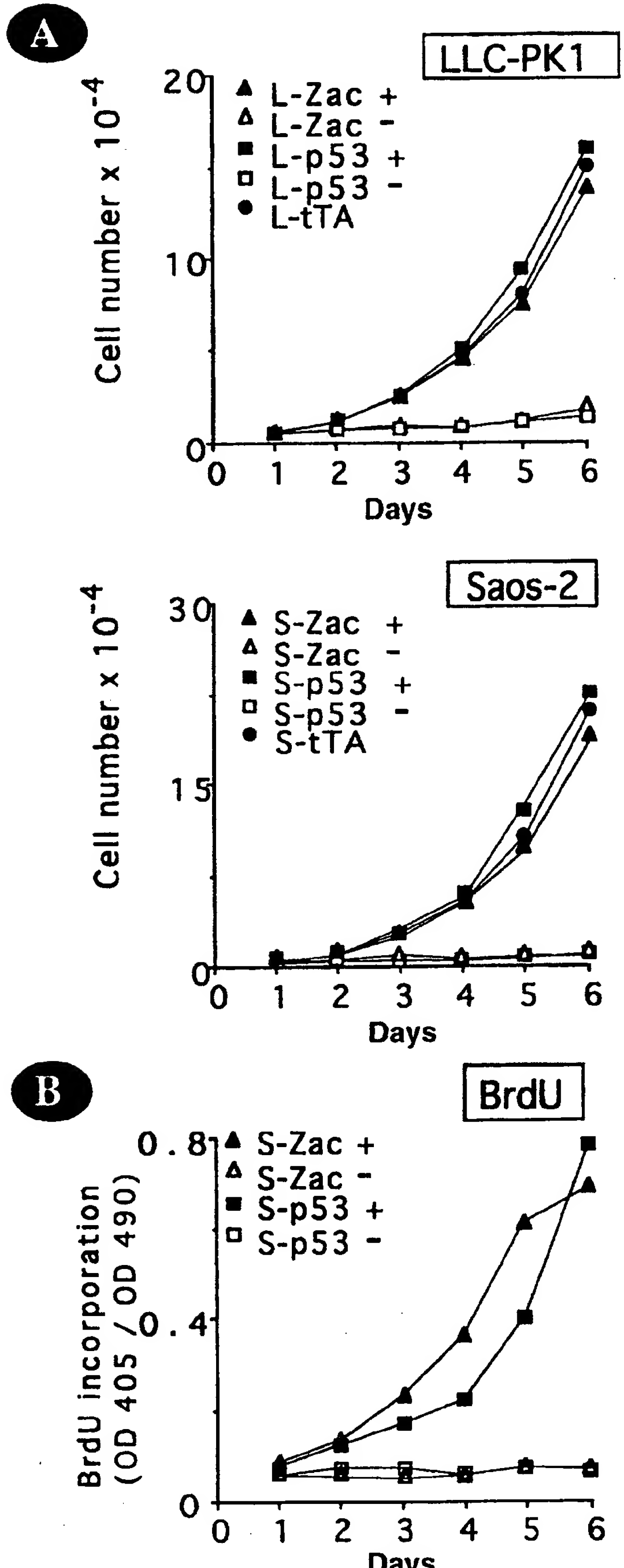

A
LLC-PK1
L-Zac +
L-Zac -
L-p53 +
L-p53 -
L-tTA
Cell number x 10-4
Days
Saos-2
S-Zac +
S-Zac -
S-p53 +
S-p53 -
S-tTA
Cell number x 10-4
Days
B
BrdU
S-Zac +
S-Zac -
S-p53 +
S-p53 -
BrdU incorporation (OD 405 / OD 490)
Days

Figure 2

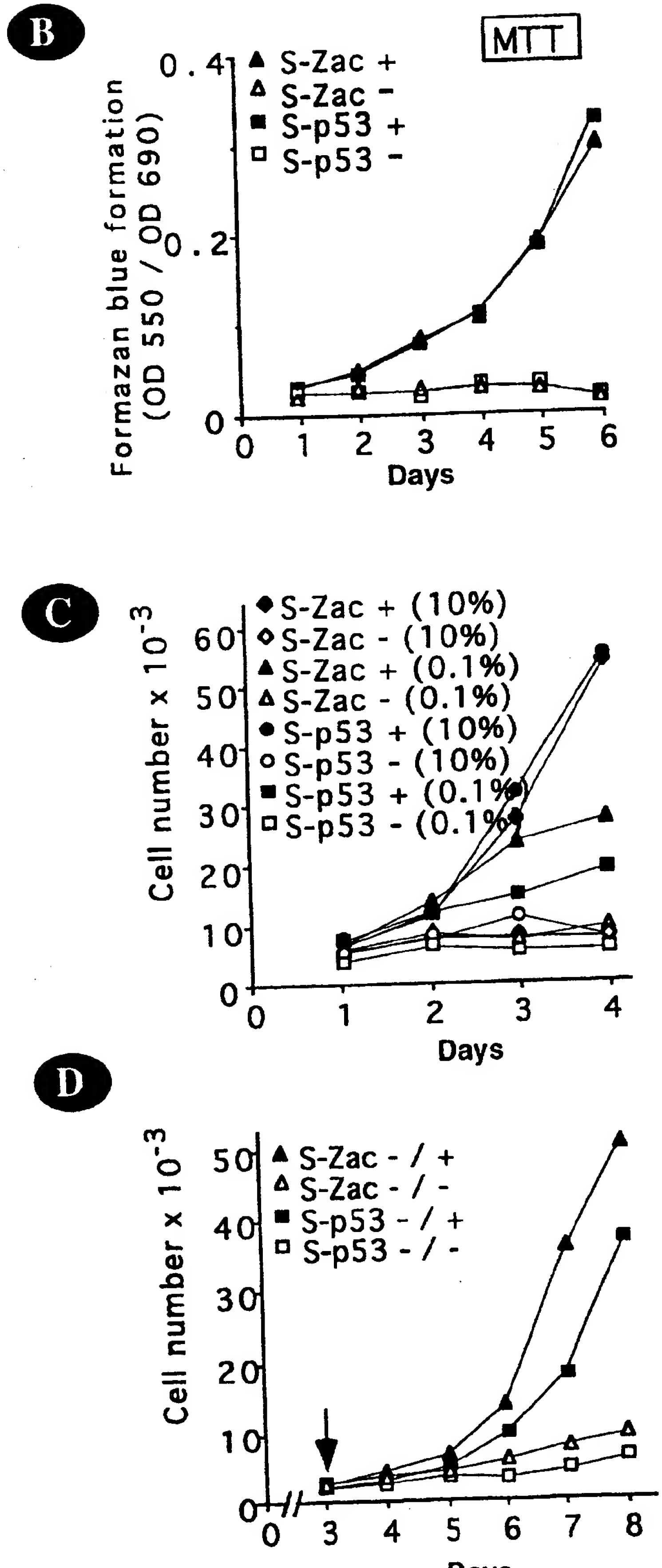


Figure 2 cont'

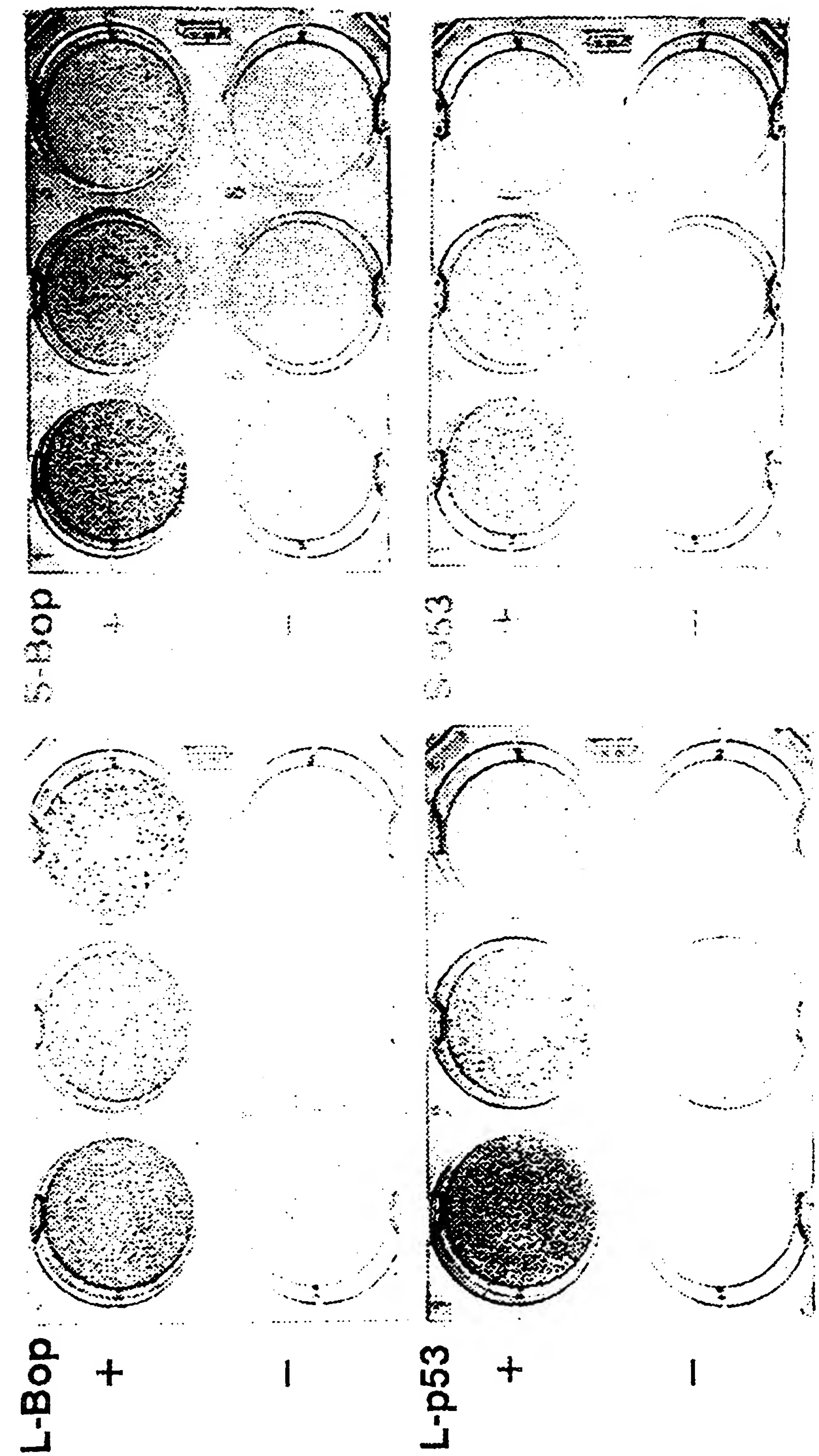


Figure 3

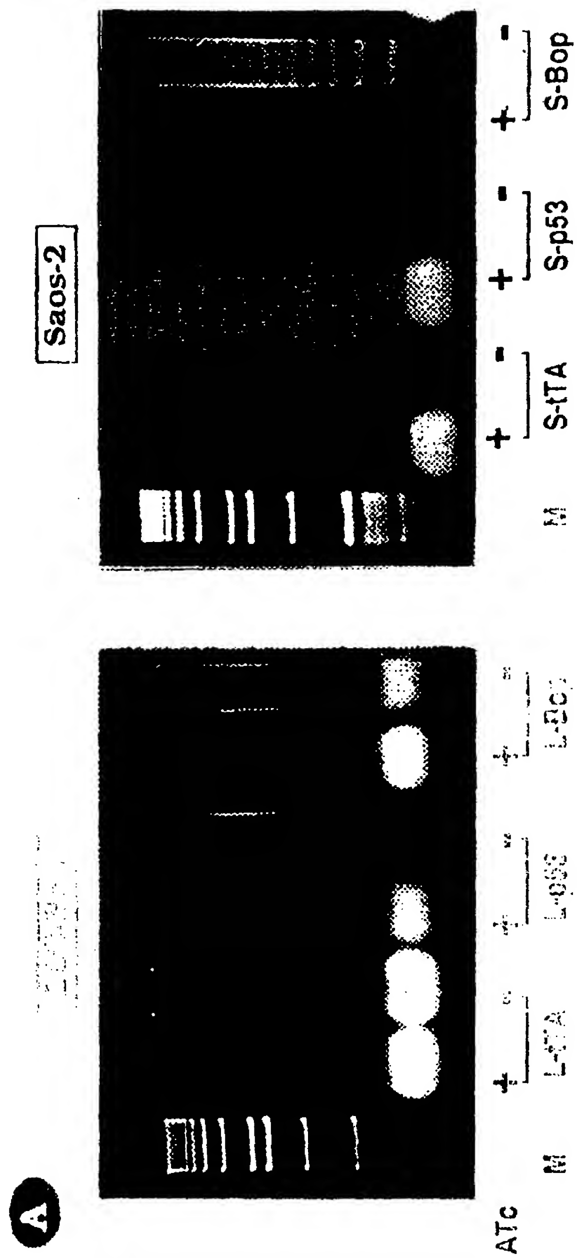
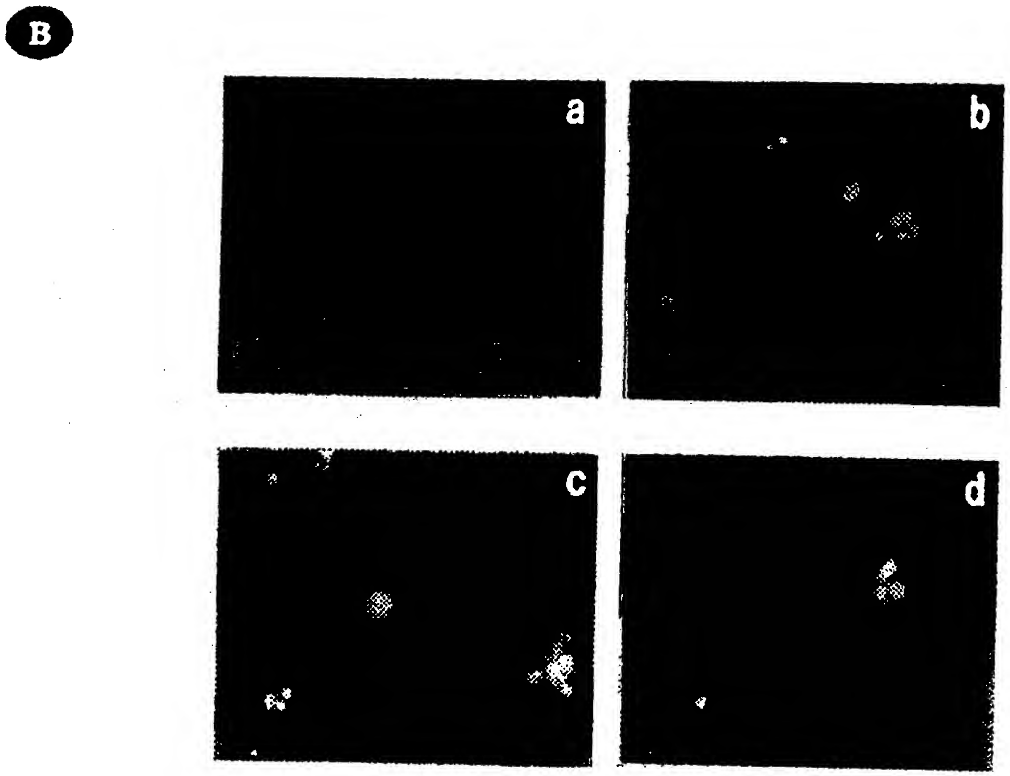
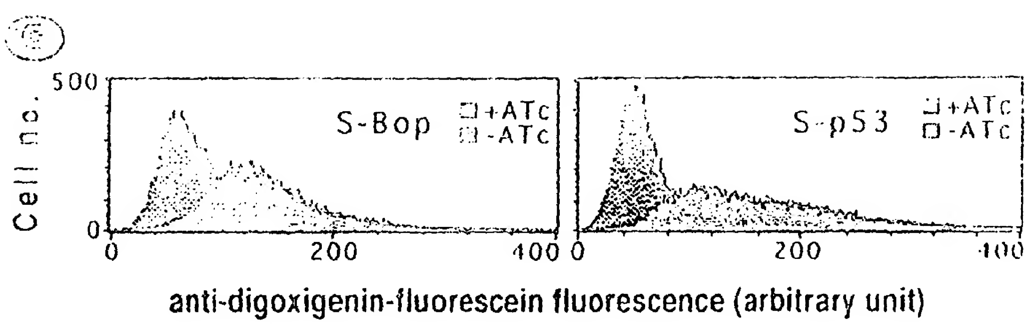


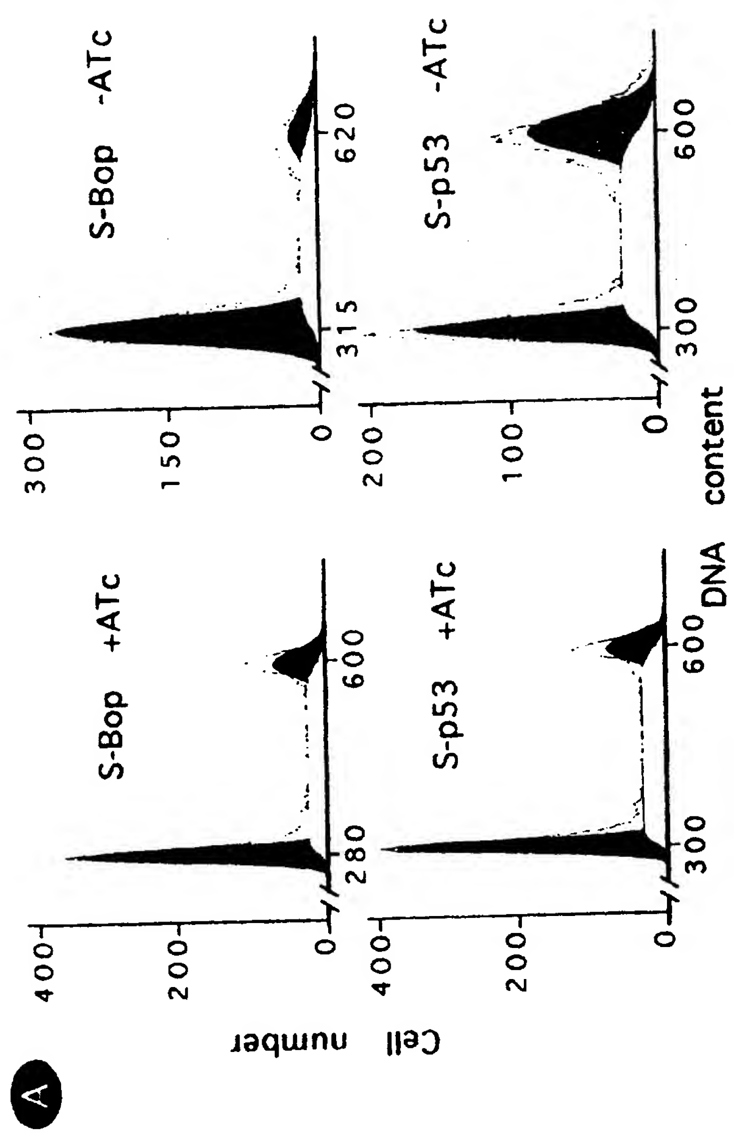
Figure 4 A



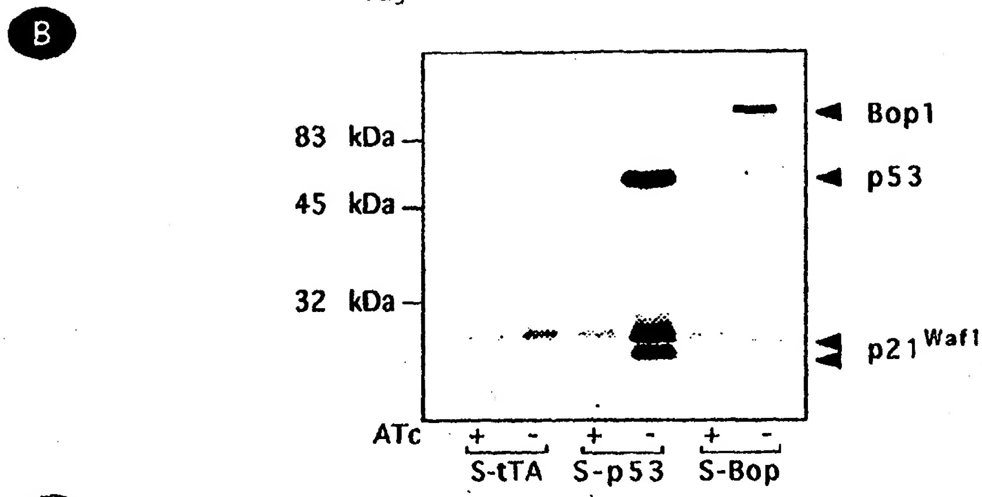


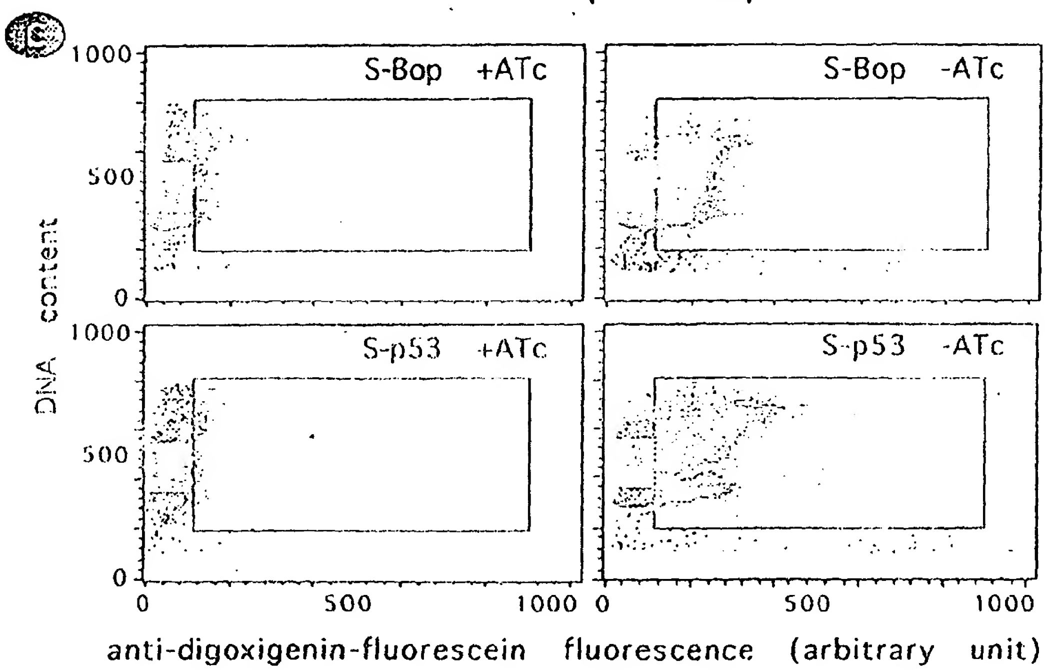
Figures 4B and 4C

Figure 5 A

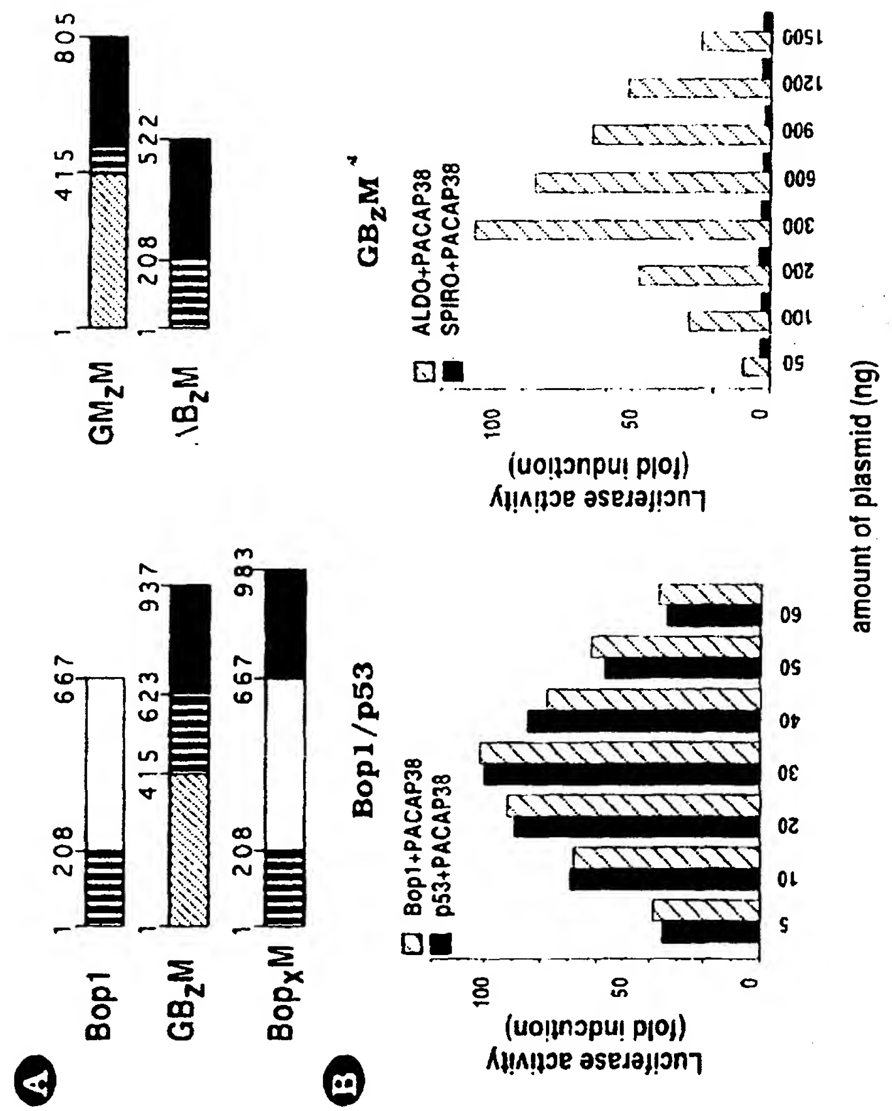

A
Cell number
S-Bop +ATc
400
200
0
280
600
S-p53 +ATc
400
200
0
300
600
S-Bop -ATc
300
150
0
315
620
S-p53 -ATc
200
100
0
300
600
DNA content

Figures 5 B and C

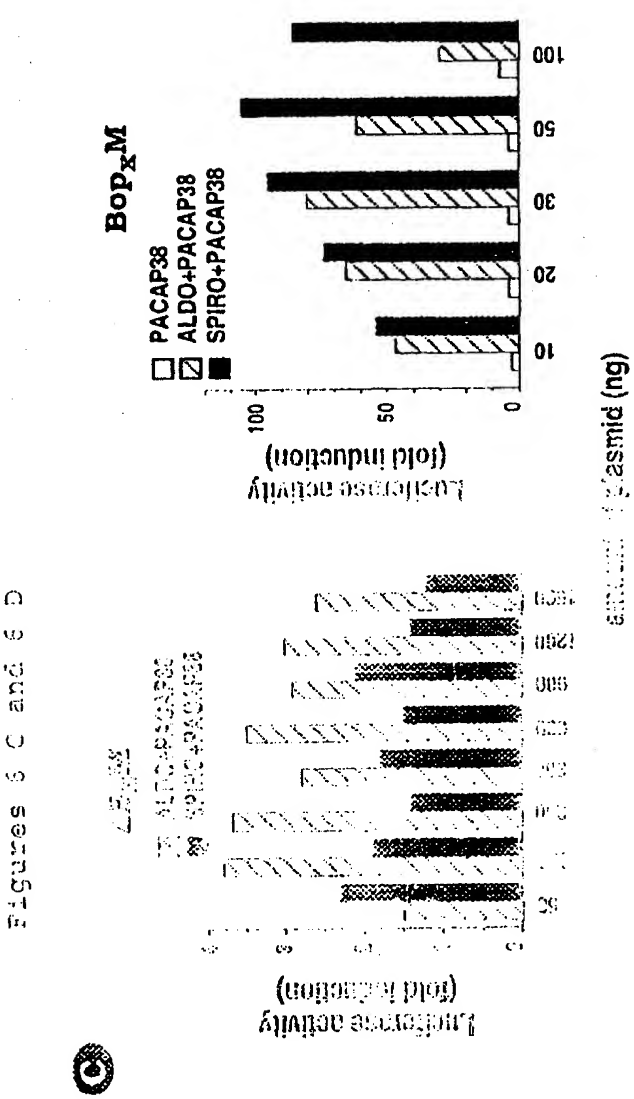




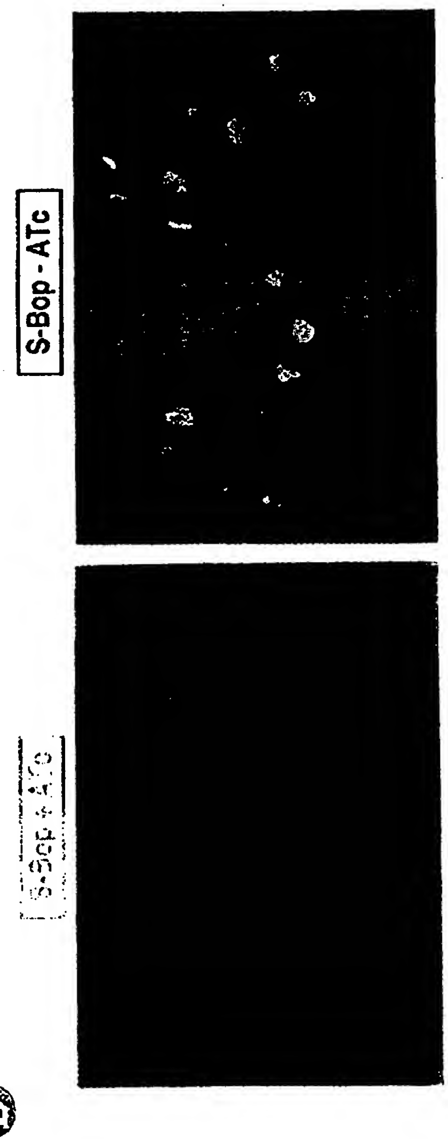
Figures 6 A and 6 B



C


Bop$_x$M
PACAP38
ALDO+PACAP38
SPIRO+PACAP38
Luciferase activity
(fold induction)
100
50
0
10
20
30
50
100
plasmid (ng)

D


S-Bop - ATc
S-Bop + ATc

(A)

```
  1 MAPFRCQKCGKSFVTLEKFTIHNYSHSRERPFKCSKAECGKAFVSKYKLMRHMATHSPQKIHQCTHCEKTFNRKDHLKNH mZAC
  1 MAPFRCQKCGKSFLTLEKFTIHNYSHTRERPFKCSKTECGKAFVSKYKLMRHMATHSPQKTHQCTHCEKTFNRKDHLKNH rLOT
  1 MATFPCQLCGKTFLTLEKFTIHNYSHSRERPYKCVQPDCGKAFVSRYKLMRHMATHSPQKSHQCAHCEKTFNRKDHLKNH hZAC

 81 LQTHDPNKISYACDDCGKKYHTMLGYKRHLALHSASNGDLTCGVCTLELGSTEVLLDHLKSHAEEKANQAPREKKYQCDH mZAC
 81 LQTHDPNKMIYACEDCGKKYHTMLGYKRHMALHSASSGDLTCGVCTLELGSTEVLLDHLKSHAEEKAHHAPREKKHQCDH rLOT
 81 LQTHDPNKMAFGCEECGKKYNTMLGYKRHLALHAASSGDLTCGVCALELGSTEVLLDHLKAHAEEKPPSGTKEKKHQCDH hZAC

161 CDRCFYTRKDVRRHLVVHTGCKDFLCQFCAQRFGRKDHLTRHTKKTHSQELMQENMQAGDYQSNEQLIAPSTSFQIKVDP mZAC
161 CERCFYTRKDVRRHLVVHTGCKDFLCQFCAQRFGRKDHLTRHTKKTHSQELMQESLQAGEYQGGYQPIAPP--FQIKADP rLOT
161 CERCFYTRKDVRRHLVVHTGCKDFLCQFCAQRFGRKDHLTRHTKKTHSQELMKESLQTGDLLSTFHTISPS--FQLKAAA hZAC

241 MPPFQLGAAPENGLDGGLPPEVHGLVLAAPEEAEQPMPPLEPLEPLEPLEPLEPMQSLEPLQPLEPMQPLEPMQPLEPMQ mZAC
239 MPPFQLEMPPESGLDGGLPPEIHGLVLASPEEVPQPM------------------------------------------ rLOT
239 LPPFPLGASAQNGLASSLPAEVHSLTLSPPEQAAQPMQPL--------------------------------------- hZAC

321 PLEPLEPLEPMQPLEPMQPLEPMQPMLPMQPMQPMQPMQPMLPMQPMLPMQPMQPMQPMLPMPEPSFTLHPGVVPTSPPP mZAC
276 --------------------------LSMPPMQPM---------------------------PEQPFTLHPGVVPSSPPP rLOT
279 --------------------------------------------------------------PESLASLHPSVSPGSPPP hZAC

401 IILQEHKYNPVPTSYAPFVGMPVKADGKAECNVGFFEEFPLQEPQAPLKFNPCFEMPMEGFGKVTLSKELLVDAVNIAIP mZAC
303 IILQEHKYSPVPTSFAPFVSMPMKADLKGFCNMGLFEEFPLQECQSPVKFSQCFEMAKEGFGKVTLPKELLVDAVNIALP rLOT
297 P-LPNHKYNTTSTSYSPLASLPLKADTKGFCNISLFEDLPLQEPQSPQKLNPGFDLAKGNAGKVNLPKELPADAVNLTIP hZAC

481 ASLEISSLLGFWQLPPPTPQNGFVNSTIPVGPGEPLPHRITCLAQQQPPPLPPPPPLPLPQPLPVPQPLPQPQMQPQFQL mZAC
383 GSLEISSLLGFWQLPPPPPQNGFMNGTIPVGAGEPLPHRITCLAQQQPPPLLPPPP-----PLPLPEPLPQPQLPPQFQL rLOT
376 ASLDLSPLLGEWQLPPPATQNTEGNSTLALGPGESLPHRLSCLGQQQ--------------------------------- hZAC

561 QIQPQMQLPQLLPQLQPQQQPDPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEEEQEEAEEEAEEGAEEGAEPEAQAEE mZAC
458 QLQPQ-------PQMQPQMQLQPLQLQLPQLLPQLQPEPEPEPEPEPEEEEEEEEEIEEEEEIEEEEEAEPEAEEEEEEAEDE rLOT
423 -------------------------------------------------------------------------------- hZAC

641 EEEEEEAEEPQPEERAQIAVSAVNLGQPPLPPTPHIFTAGSNTAILPHFHHAFR mZAC
531 EEAEEEEEEPQPEERAQIAMSAVNMGQPPLPPTPHVFTAGTNTAILPHFHHAFR rLOT
423 ------------QEPPLAMGTVSLGQLPLPPIPHVFSAGTGSAILPHFHHAFR hZAC
```

Figure 7

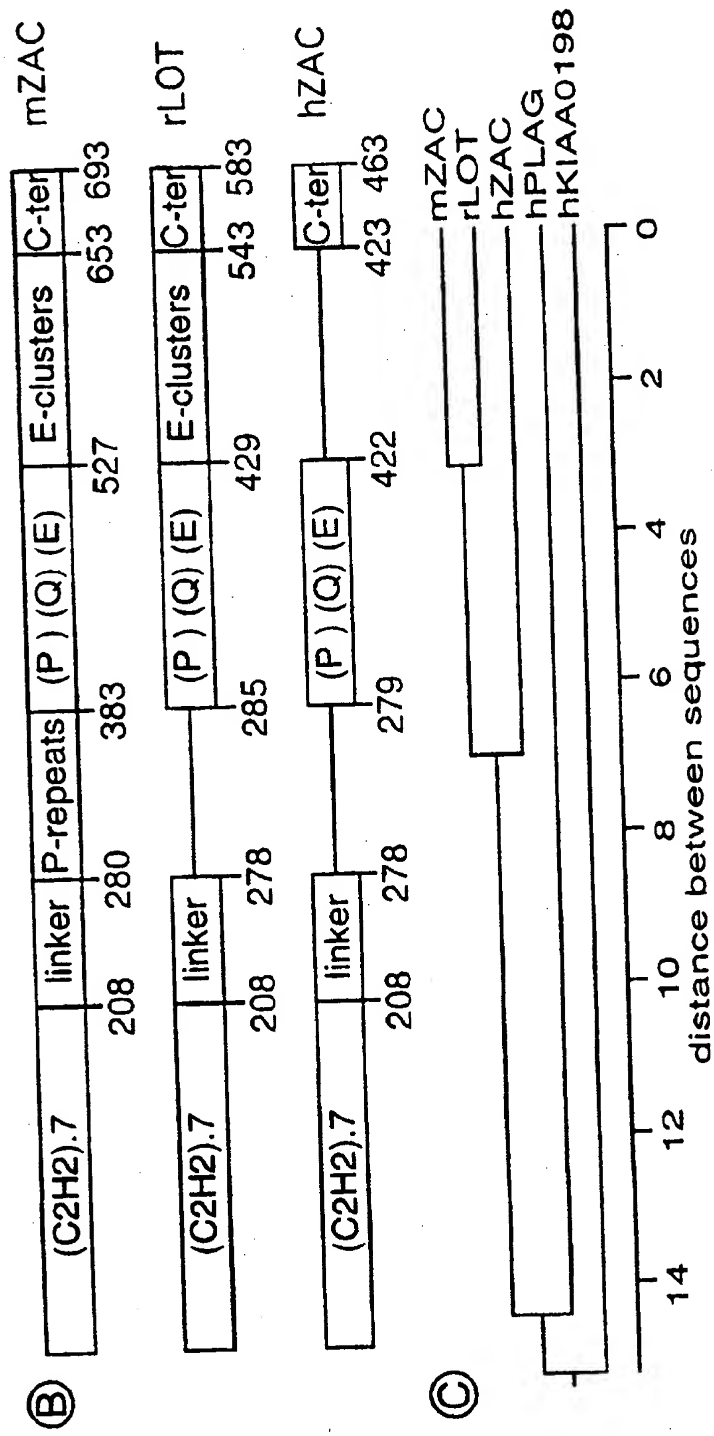


Figure 7 cont'

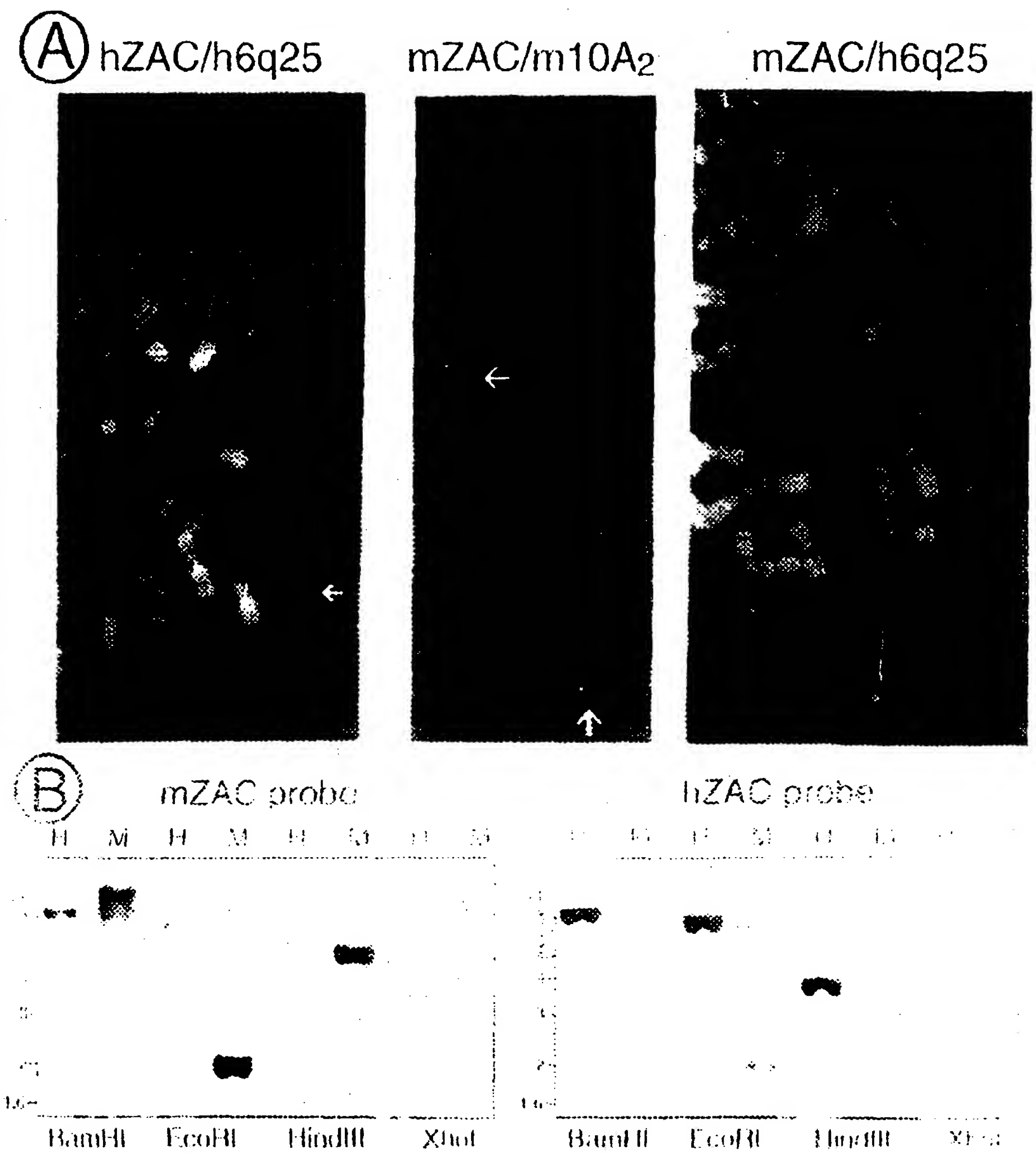

A
hZAC/h6q25
mZAC/m10A2
mZAC/h6q25
B
mZAC probe
hZAC probe
BamHI
EcoRI
HindIII
XhoI

Figure 8

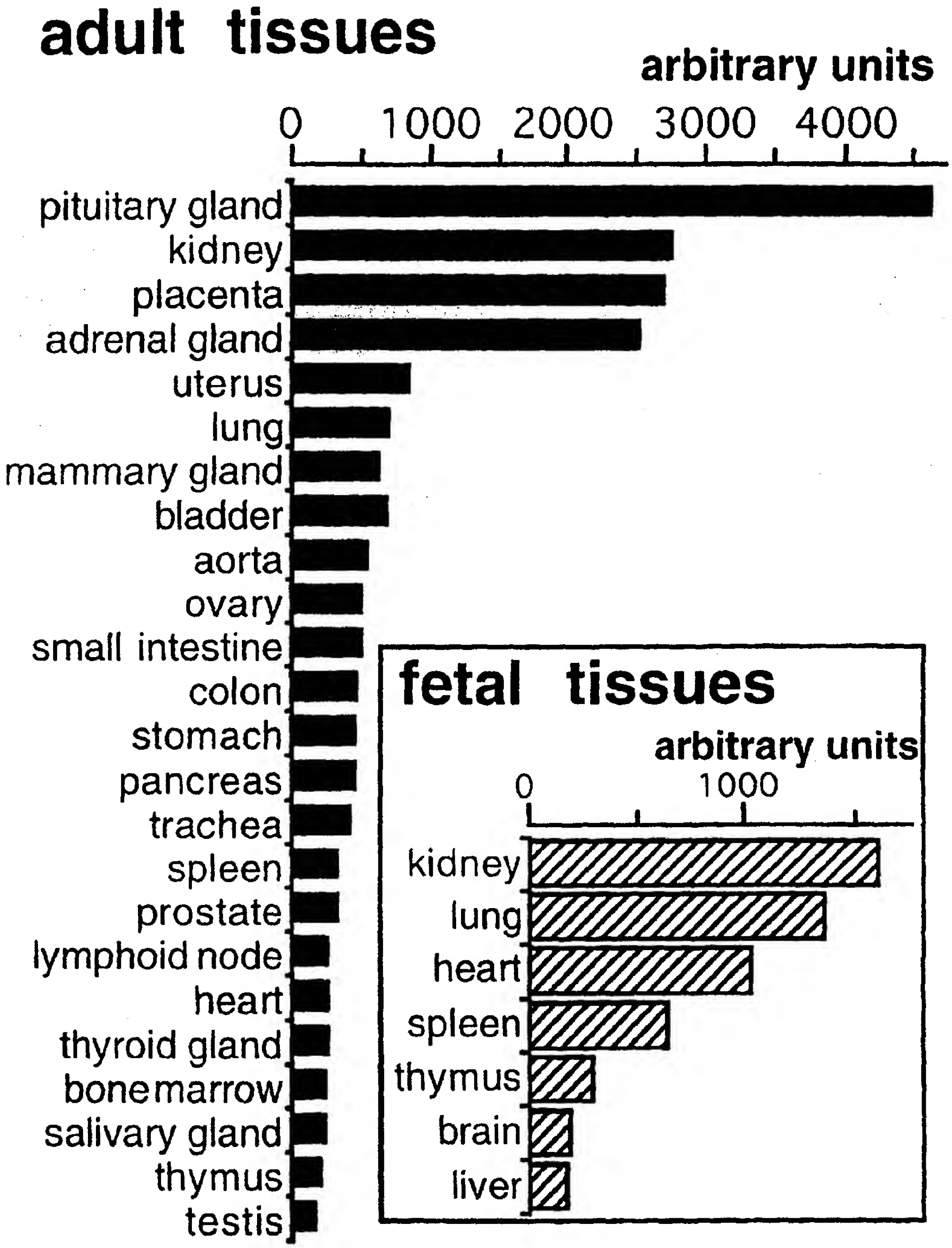

adult tissues
arbitrary units
0
1000
2000
3000
4000
pituitary gland
kidney
placenta
adrenal gland
uterus
lung
mammary gland
bladder
aorta
ovary
small intestine
colon
stomach
pancreas
trachea
spleen
prostate
lymphoid node
heart
thyroid gland
bone marrow
salivary gland
thymus
testis
fetal tissues
arbitrary units
0
1000
kidney
lung
heart
spleen
thymus
brain
liver

Figure 9

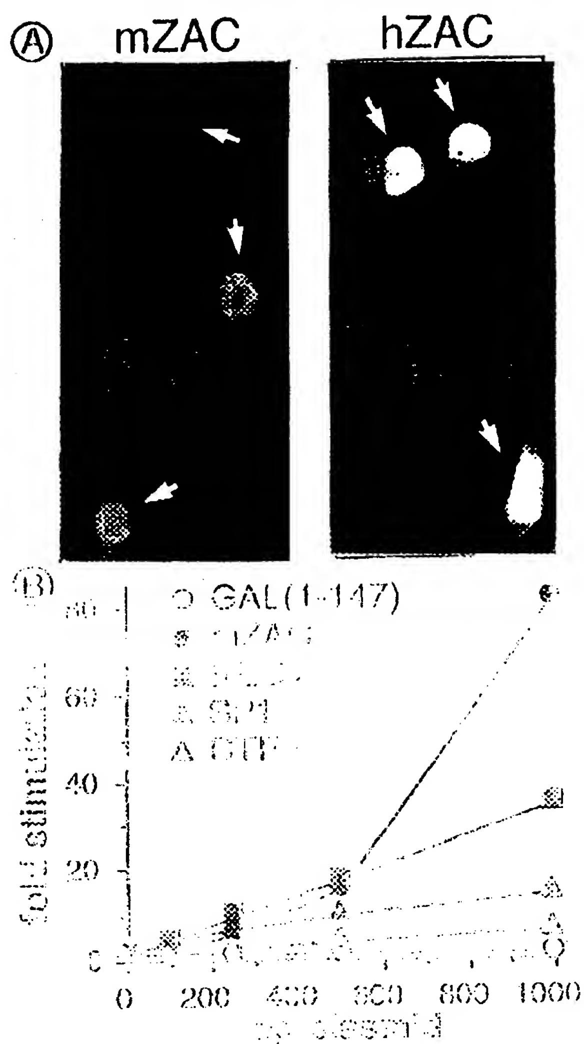


Figure 10

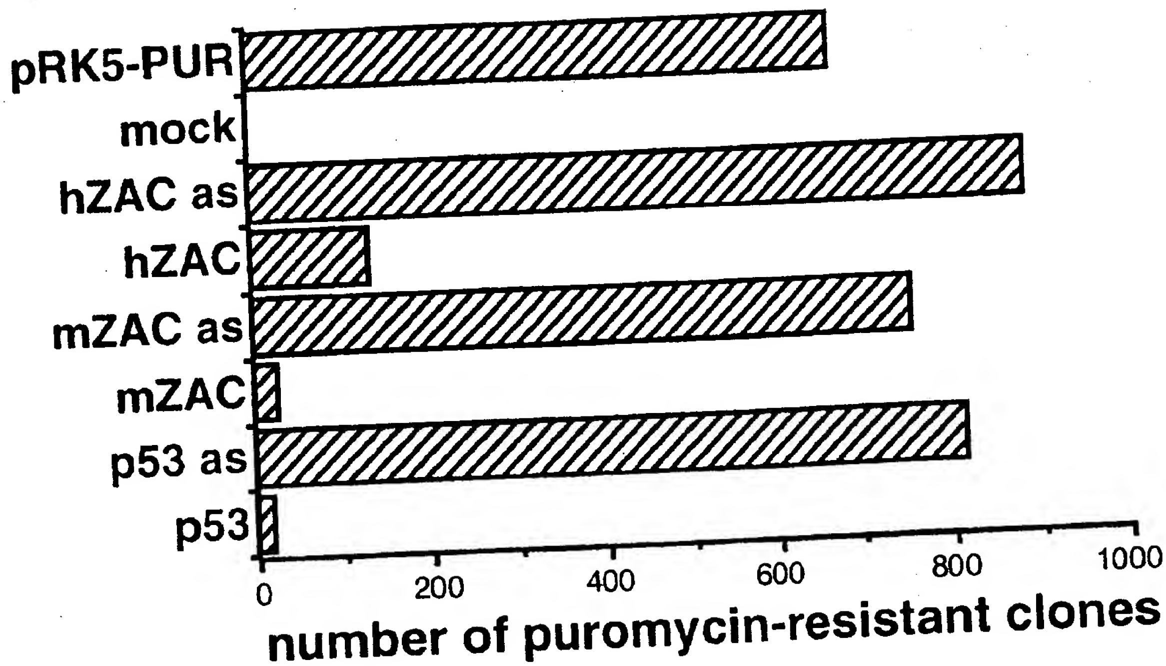

pRK5-PUR
mock
hZAC as
hZAC
mZAC as
mZAC
p53 as
p53
0
200
400
600
800
1000
number of puromycin-resistant clones

Figure 11

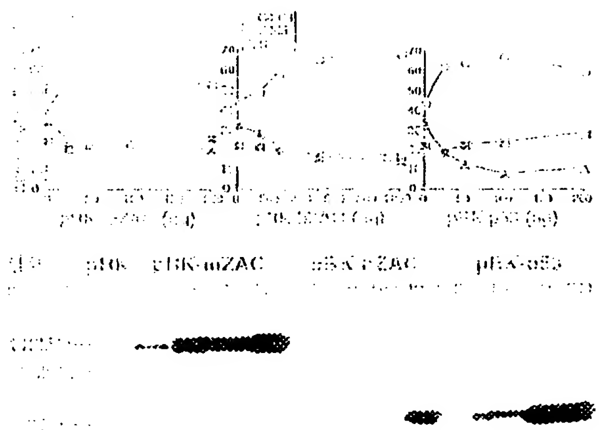


Figure 12

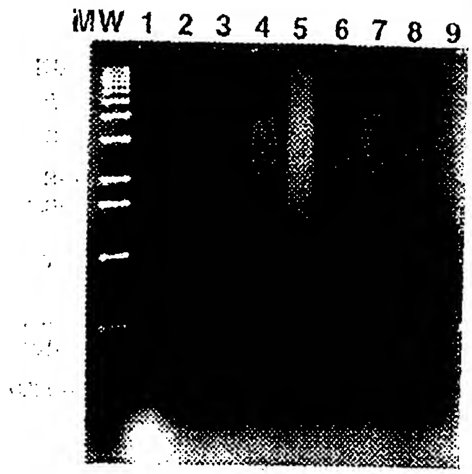


Figure 13

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/05198

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C12N15/85 C07K16/18 C07K16/32
C12N5/10 A61K38/17 G01N33/577 C12Q1/68 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 25429 A (MYRIAD GENETICS INC ;KAMB ALEXANDER (US)) 28 September 1995 see abstract see page 4 see page 113 - page 124 ---	1,11-16, 18-32, 36-43, 45-48
X	EP 0 710 722 A (UNIV CALIFORNIA) 8 May 1996 see the whole document ---	1,11-16, 18-32
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 1998	20.02.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 21734 A (US HEALTH) 18 July 1996 see page 33 - page 38 see page 81 - page 87 ---	1,11, 13-16, 18-22, 24-32, 36,37, 40-46
X	WO 95 09916 A (RHONE POULENC RORER SA ;MALLET JACQUES (FR); REVAH FREDERIC (FR);) 13 April 1995 see the whole document ---	1,12-16, 18,19, 25,27, 33-35, 40-43,45
X	DATABASE EMBL-EMEST9, RELEASE 36 Accession No. T08483, 6 August 1993 ADAMS, M.D. ET AL.: "EST06374 Homo sapiens cDNA clone HIBBE91 5' end similar to Zinc finger protein family" XP002052649 see abstract	1,13-16, 18-22
X	& ADAMS, M.D., ET AL.: "Rapid cDNA sequencing (expressed sequence tags) from a directionally cloned human infant brain cDNA library" NATURE GENETICS, vol. 4, 1993, pages 373-380, XP000604995 see the whole document ---	1,13-16, 18-22
X	SPENGLER, D. ET AL.: "Differential signal transduction by five splice variants of the PACAP receptor" NATURE, vol. 365, 9 September 1993, pages 170-175, XP002052647 cited in the application see the whole document ---	2-10
P,X	SPENGLER, D. ET AL.: "Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain" THE EMBO JOURNAL, vol. 16, no. 10, 15 May 1997, pages 2814-2825, XP002052648 see the whole document ---	1-26,28, 36,37

-/--

2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/05198

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ABDOLLAHI A ET AL: "IDENTIFICATION OF A GENE WHICH SHOWS DECREASED EXPRESSION IN MALIGNANTLY TRANSFORMED RAT OVARIAN SURFACE EPITHELIAL CELLS" PROCEEDINGS OF THE 87TH. ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, WASHINGTON, APR. 20 - 24, 1996, vol. 37, 20 April 1996, AMERICAN ASSOCIATION FOR CANCER RESEARCH, page 242 XP002036823 see the whole document -----	1,11-25

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT	International application No. PCT/EP 97/05198

Box I Observations where certain claims w r found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 97/05198

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 29-35 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 97/05198

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9525429 A	28-09-95	AU 2186395 A	09-10-95
		CA 2162147 A	28-09-95
		CN 1128935 A	14-08-96
		EP 0708592 A	01-05-96
		FI 955415 A	09-01-96
		JP 8510651 T	12-11-96
		NO 954494 A	17-01-96
		AU 2378995 A	09-10-95
		CA 2162150 A	28-09-95
		CN 1128049 A	31-07-96
		EP 0702730 A	27-03-96
		FI 955416 A	09-01-96
		JP 8510391 T	05-11-96
		NO 954495 A	17-01-96
		NZ 284612 A	22-09-97
		WO 9525813 A	28-09-95
		US 5624819 A	29-04-97
EP 0710722 A	08-05-96	AT 136793 T	15-05-96
		AU 8183694 A	23-02-95
		AU 653356 B	29-09-94
		AU 8262991 A	27-02-92
		CA 2047090 A	25-02-92
		DE 69118789 D	23-05-96
		DE 69118789 T	26-09-96
		EP 0475623 A	18-03-92
		ES 2085966 T	16-06-96
		JP 7059579 A	07-03-95
		KR 9700189 B	06-01-97
		US 5532220 A	02-07-96
WO 9621734 A	18-07-96	AU 4755496 A	31-07-96
		CA 2209990 A	18-07-96
WO 9509916 A	13-04-95	FR 2710846 A	14-04-95
		AU 7816294 A	01-05-95
		CA 2173338 A	13-04-95
		EP 0722496 A	24-07-96
		FI 961494 A	03-04-96
		JP 9503915 T	22-04-97

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 97/05198

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9509916 A		NO 961220 A	26-03-96

Form PCT/ISA/210 (patent family annex) (July 1992)